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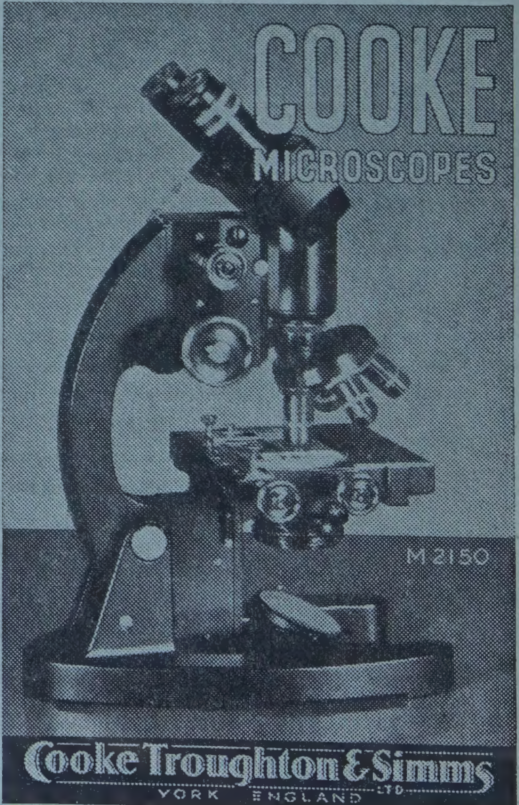

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ENVIRONMENTAL FACTORS INFLUENCING THE INDUCTION AND TERMINATION OF DIAPAUSE IN THE EMPEROR DRAGONFLY, *ANAX IMPERATOR* LEACH (ODONATA: AESHNIDAE)

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(*Received* 6 *June* 1955)

INTRODUCTION

The terminology and physiological interpretation of diapause used in this paper are those set forth by Andrewartha (1952).

Field observations on *Anax imperator* (Corbet, 1954, 1955, 1956) have shown that, although final instar larvae may be present at all times of the year, emergence of the adult is restricted to a period of about 6 weeks, between mid-May and mid-July. Larvae entering the final instar in spring proceed directly to metamorphosis, whereas those doing so after the end of May undergo delayed development, and do not metamorphose until the following spring. This period of delayed development persists when environmental conditions are otherwise suitable for metamorphosis; it can therefore be regarded as a diapause stage. The fact that diapause is associated with larvae which enter the final instar after 1 June, but not with those doing so before, indicates that it is facultative and not an obligate feature of the life history.

In several cases of insects exhibiting facultative diapause, the environmental factor inducing diapause is absolute day length (e.g. *Bombyx*, Kogure, 1933; *Laspeyresia*, Dickson, 1949; *Diataraxia*, Way & Hopkins, 1950). In *Anax*, however, this is clearly not the major factor involved in the response, since photoperiods characteristic of April and May ('non-diapause months') occur also in September and August ('diapause months'). This seasonal distribution of diapause suggests that the environmental factor responsible for its determination may be the sign of the change in successive lengths of day. Certain birds and mammals in which such a response has been recognized (Rowan, 1938; Bissonnette, 1936; Marshall, 1937) share with *Anax* the ecological need to maintain an annual cycle during a life exceeding 1 year. Previous studies of insect diapause, on the other hand, have involved species having life histories of 1 year or less.

Preliminary experiments designed to investigate certain aspects of this hypothesis are described below. Time available for their design was severely limited, and it was not possible to maintain a constant temperature entirely independent of photoperiod. This is an important weakness in the experimental design which will need to be rectified in any further work. Theoretical problems were encountered associated with analysis of the effects of changing, rather than absolute, photoperiod, and these also have tended to reduce the rigour of some of the controls. In addition, owing to the difficulty of obtaining adequate material at the appropriate season, the number of larvae in each group was small. Conclusions which can be drawn from these experiments are therefore limited and provisional. Nevertheless, the results provide strong indications of the pattern of larval responses to the environmental factors concerned, and should reduce the scope of further analyses of this example of facultative diapause.

MATERIAL AND METHODS

Larvae were obtained from the Fish Pond, Wokefield Common, Berkshire, the habitat where most of the field observations on the life history were made (Corbet, 1956). They were kept in rectangular, white enamel, hospital trays (10 × 8 × 2 in.) fitted with wooden frames made from strips of dowelling. The latter satisfied the thigmotactic requirements of larvae and enabled them to emerge without difficulty. The trays were kept filled with tap water.

Live earthworms were provided as food, at a rate approximating to one worm (3 in. long) per larva every other day. It was not possible to standardize feeding without confining each larva in a separate container, a procedure for which space was not available. However, there can be little doubt that larvae were receiving adequate food, since several worms usually remained untouched in each container after each occasion of feeding.

When it was necessary to control photoperiod artificially, trays containing larvae were housed in wooden, light-proof boxes, each with a lighting unit in the lid. Light was provided by two 2 ft., fluorescent (daylight) tubes (Mazda 20 W. 'natural') in each box. These gave a total light intensity varying between 100 and 200 foot-candles in the region occupied by the trays. Photoperiodic regime was controlled by the use of Venner mercury-break time-switches and Venner Solar Dial time-switches. In the latter type, by exchanging the 'ON' and 'OFF' levers, it was possible to reproduce normal seasonal sequences of photoperiods; a 'calendar dial' enabled the date of setting to be controlled.

There was no facility for maintaining a constant temperature entirely independent of photoperiod. Constant-temperature rooms were used when possible, but, especially in summer, the lighting units in the boxes raised the temperature beyond the limit prescribed for the rooms and thus prohibited their use. Throughout the experiments, however, control groups were kept in the same room, and therefore temperature differences were slight and due to photoperiod alone.

The end-point chosen to specify the onset of metamorphosis (regarded here as post-diapause development) was the first mesial meeting of the compound eyes. This corresponds to the first day of stage (4) of metamorphosis (see Corbet, 1956), and, at about 20° C., occurs about 14 days after the first visible signs of metamorphosis and about 17 days before emergence. The time elapsing from ecdysis (to the final instar) to the onset of metamorphosis was regarded as the indication of whether or not a larva had entered diapause. In diapause larvae, at about 23° C., this time was about 105 days; in non-diapause larvae it was about 30 days.

When larvae with different histories were kept in the same tray, it was necessary to distinguish them as individuals. This was done by marking them on the legs with strips of coloured tin foil, according to the method described elsewhere (Corbet, 1956).

EXPERIMENTAL DESIGN AND RESULTS

The abbreviation adopted to indicate photoperiod is such that 04.00 denotes 4 hr. light and 20 hr. darkness per day, and 22.16 denotes 22 hr. 16 min. light and 1 hr. 44 min. darkness per day.

No larvae amongst the experimental material showed any signs of metamorphosis at the time of collection.

Experiment 1 (17 July 1952 to 12 April 1953)

Objects. To determine the effect of changing photoperiod upon the induction of diapause; to determine the larval stage in which diapause is evoked, and to determine the effect of temperature on the completion of diapause development.

Material. Penultimate instar: 110 larvae (49♂, 61♀) collected on 16 July 1952; 6 larvae (4 ♂, 2 ♀) collected on 25 August 1952. (Due to cannibalism during ecdysis, only 75 (36 ♂, 39 ♀) of these larvae survived the experiments to provide results.)

Final instar: 9 larvae (8 ♂, 1 ♀) collected on 16 July 1952 for use in groups A6 and C3 (see Fig. 2); 12 larvae (6 ♂, 6 ♀) collected on 25 August 1952 for use in group A7.

Conditions. Four sets of experimental conditions were set up as follows (regimes of photoperiod are made clear in Fig. 1):

Box A. Average temperature about 23° C.; range 18–30° C. Natural photoperiods decreasing from 16.08 (17 July) by about 3 min. per day.

Box B. Average temperature about 6° C.; range 1–9° C. Photoperiods similar to Box A.

Box C. Average temperature about 25° C.; range 20–30° C. Photoperiods increasing in length from 16.08 by regular increments of 3 min. per day, reaching 24.00 on 21 December.*

Box D. Average temperature about 24° C.; range 20–30° C. Photoperiods constant at 15.30 (31 July).

The four main experimental groups were subdivided as shown in Fig. 2, and the numbers of larvae which survived to provide results can be determined from Appendix 1.

---

* *Box C.* There are difficulties inherent in devising these conditions which cannot be overcome save by using larvae reared in captivity. In the field, larvae in the penultimate instar are only present in adequate numbers in July and August. If photoperiods are increasing by appreciable increments from values characteristic of these months, they soon exceed the longest photoperiod which can be experienced in nature. The alternative course of making an initial reduction in the photoperiod might induce diapause and thus counteract the effect of subsequent treatment. (It may be noted that this objection was not overcome in the original experiments stimulating maturation of the gonads in the junco (Rowan, 1925, 1926, 1928).)

Results. These are summarized in Tables 1–3 and are given in detail in Appendix 1.

Box A1 (Table 1). All larvae entered diapause except 1 ♀. This delayed ecdysis until 24 January and was therefore the only individual not experiencing decreasing photoperiods throughout the final instar. After completion of diapause development, metamorphosis occurred normally under decreasing photoperiods.

Box A2. See box C2.

Box A3. See box D2.

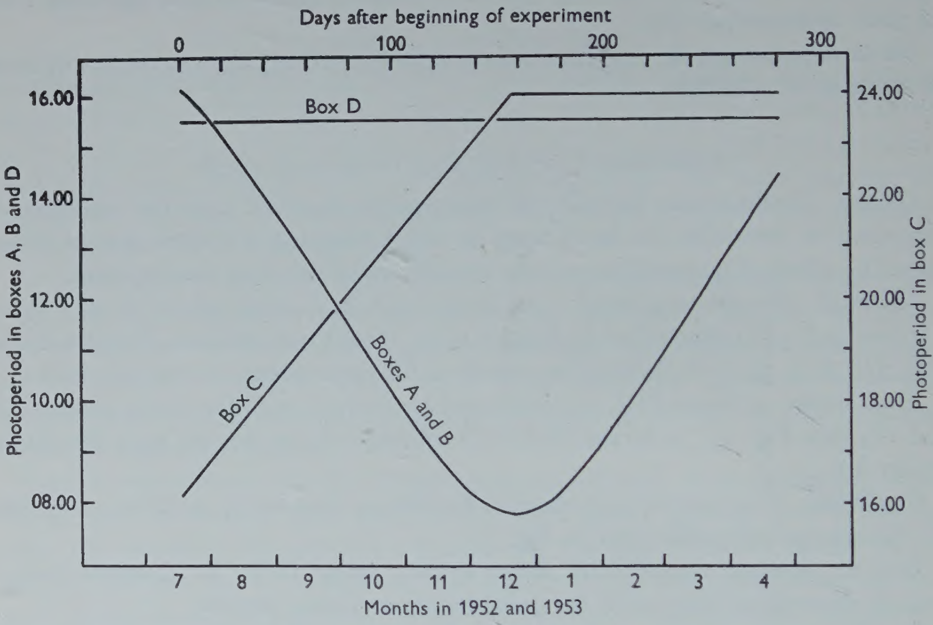

Fig. 1. Exp. 1. Photoperiodic regime.

Box A4 (Table 1). Only two larvae failed to enter diapause. These delayed ecdysis until 17 and 19 February and thus experienced increasing photoperiods throughout the final instar. All other larvae moulted before the winter solstice; for these, the time required for completion of diapause development was only reduced slightly after exposure to low temperature, but variation was less than in A1. While photoperiods were decreasing, down to 08.00, larvae continued to enter diapause.

Box A5 (Table 1). All larvae underwent diapause development after return to high temperatures. The time for its completion was slightly less than in A1, and variation was again reduced. The larva chilled for 16 weeks seemed intermediate in type between diapause and non-diapause states; this was the only larva to experience increasing photoperiods throughout its period at high temperatures.

Box A6 (Table 1). All larvae showed intense diapause.

Box A7 (Table 1). All larvae showed diapause.

Box B1, 2, 3. See boxes A4, 5, 7, respectively.

Box C 1 (Table 2). Responses of larvae were varied. Three were definitely non-diapause (metamorphosing after 24, 27 and 32 days), and the remainder seemed intermediate in type (48–82 days). Here, abnormally long photoperiods may have influenced the response to increments.

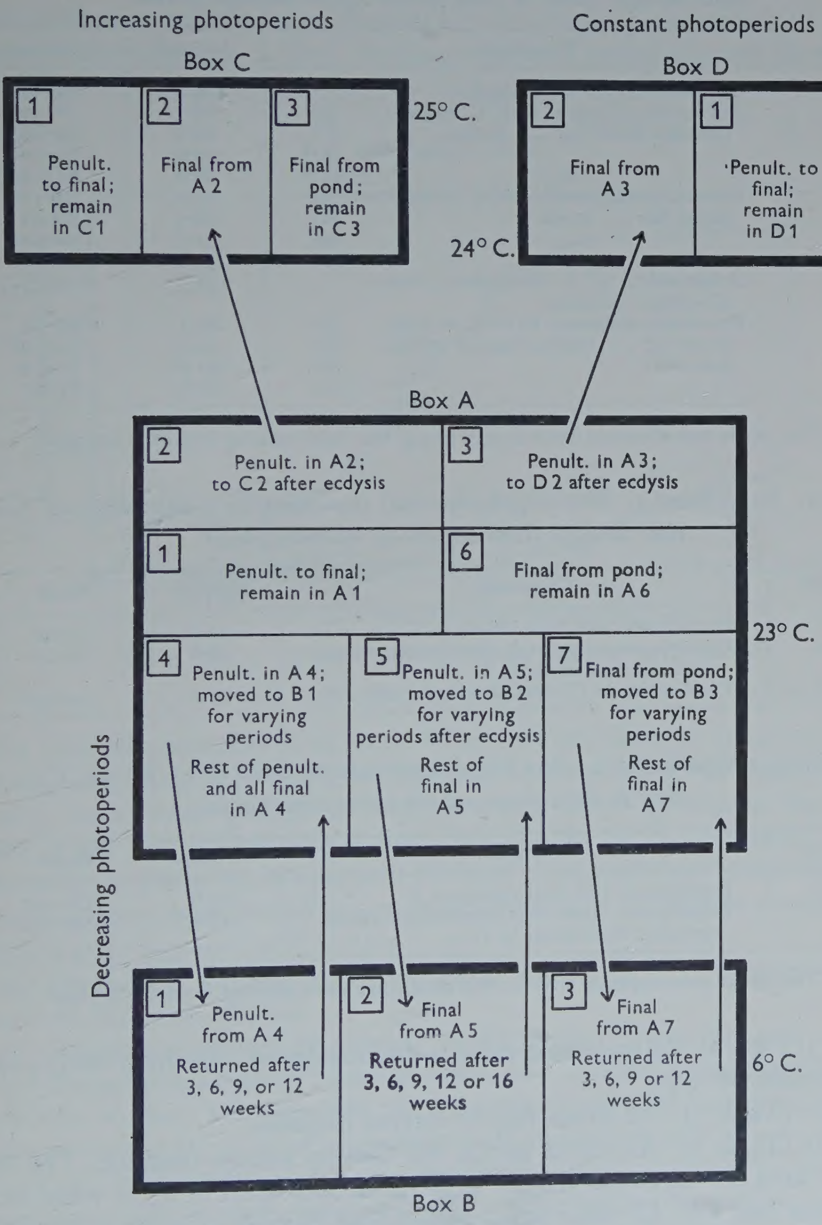
Increasing photoperiods
Box C
1
Penult. to final; remain in C 1
2
Final from A 2
3
Final from pond; remain in C 3
25° C.
Constant photoperiods
Box D
2
Final from A 3
1
Penult. to final; remain in D 1
24° C.
Box A
2
Penult. in A 2; to C 2 after ecdysis
3
Penult. in A 3; to D 2 after ecdysis
1
Penult. to final; remain in A 1
6
Final from pond; remain in A 6
23° C.
4
Penult. in A 4; moved to B 1 for varying periods
Rest of penult. and all final in A 4
5
Penult. in A 5; moved to B 2 for varying periods after ecdysis
Rest of final in A 5
7
Final from pond; moved to B 3 for varying periods
Rest of final in A 7
Decreasing photoperiods
1
Penult. from A 4
Returned after 3, 6, 9, or 12 weeks
2
Final from A 5
Returned after 3, 6, 9, 12 or 16 weeks
3
Final from A 7
Returned after 3, 6, 9 or 12 weeks
6° C.
Box B

Fig. 2. Exp. 1. Showing distribution of control groups.

Box C2 (Table 2). Responses of larvae were varied. Two were non-diapause (19 and 29 days); the remainder were intermediate in type but were closer to diapause larvae.

Table 1. *Experiment* 1. *Box A (photoperiods decreasing naturally;* 23° C.); *time in days spent in final instar before metamorphosis*

Group	Treatment		Average	Range
A1	Subjected to 23° C. throughout		104·4	65–133
A4	Previously subjected to 6° C. in penultimate instar for ... weeks	(3)	96·3	78–107
		(6)	99·5	89–105
		(9)*	92·0	84–104
		(12)*	86·0	61–109
A5	Previously subjected to 6° C. in final instar for ... weeks	(3)	93·0	85–101
		(6)	92·3	90–95
		(9)	95·3	90–104
		(12)	93·0	82–108
A6	Subjected to 23° C. throughout; date of ecdysis unknown		130·0	102–174
A7	Previously subjected to 6° C. in final instar for ... weeks; date of ecdysis unknown	(3)	87·3	75–94
		(6)	90·3	75–106
		(9)	101·6	87–122
		(12)	87·3	74–95

* The single non-diapause larva in this group has been omitted from the average.

Table 2. *Experiment* 1. *Box C (photoperiods increasing by* 3 *min./day;* 25° C); *time in days from ecdysis to metamorphosis*

Group	Treatment	Average	Range
C1	Penultimate and final instars in C	51·3	24–82
C2	Penultimate instar in A (decreasing photoperiods); final instar in C	58·8	19–91
C3	Final instar in C; date of ecdysis unknown	81·0	54–108

Table 3. *Experiment* 1. *Box D (photoperiods constant at* 15.30; 24° C); *time in days from ecdysis to metamorphosis*

Group	Treatment	Average	Range
D1	Penultimate and final instars in D	107·8	77–144
D2	Penultimate instar in A (decreasing photoperiods); final instar in D	123·6*	104–135

* The single non-diapause larva in this group has been omitted from the average.

Box C3 (Table 2). All may be regarded as diapause larvae, excepting one (54 days) of an intermediate type.

Box D1 (Table 3). All larvae showed intense diapause.

Box D2 (Table 3). All larvae except one showed intense diapause. The non-diapause larva (27 days) was subject to an initial increment of 03.57 when transferred from box A3. All other larvae experienced changes of 00.21 or less at the time of moving.

Ecdysis to the final instar (boxes A, C 1, and D 1). Records for dates of ecdysis for penultimate instar larvae in A 1, 2, 3 and 5 show that most larvae moulted at a photoperiod of 14.00–12.00 (Fig. 3). In C 1 ecdysis was greatly delayed, and in D 1 it occurred slowly.

Conclusions. (1) Diapause occurs in the final instar and takes the form of an interruption in morphogenesis such that, at about 23° C., metamorphosis does not occur until some 105 days after ecdysis (A 1). In non-diapause larvae this period is about 30 days (A 4, C 1, 2, D 2). Feeding continues during diapause development (A 1, D 1).

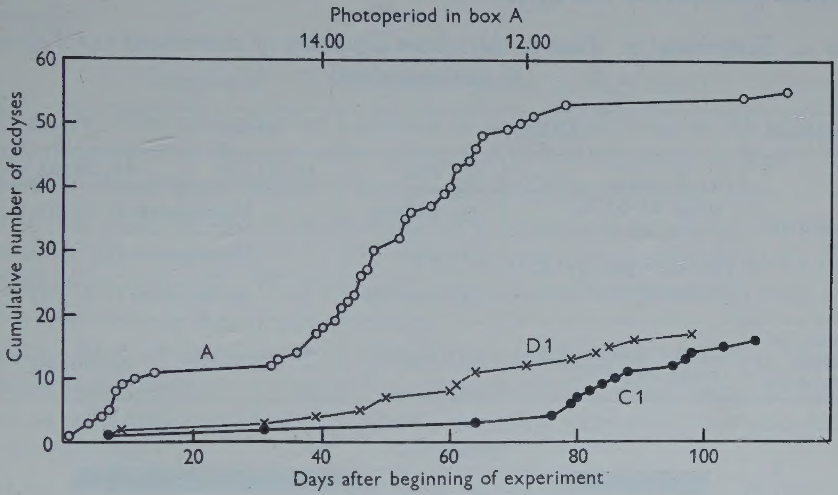

Fig. 3. Exp. 1. Ecdyses to the final instar in boxes A, C 1 and D 1.

(2) Low temperatures of about 6° C. are not necessary to complete diapause development (A 1, D 1). Negligible diapause development occurs during exposure to 6° C. for 12 weeks (A 5, 7). At temperatures of 18–30° C. diapause may proceed faster at low than at high temperatures (A 1, D 1, 2), but in the present cases it is impossible to assess the independent effects of photoperiod and temperature.

(3) Variation in the period necessary for completion of diapause development is reduced after exposure to 6° C. (A 4, 5, 7).

(4) Diapause is induced by decreasing photoperiods (A 1) irrespective of the length of day involved (A 4).

(5) Diapause is induced by constant photoperiods of 15.30 (D 1, 2).

(6) Diapause is prevented by an exposure to an increase in photoperiod immediately after ecdysis. This may take the form of a single large increase (C 2, D 2) of more than 00.21 (D 2), or of successive small increments such as occur in spring (A 4, C 1). It is possible that under unnaturally long photoperiods larvae do not respond normally to regular increments (C 1, 2); in the two cases of increase within normal day lengths (A 4) there was complete absence of diapause.

(7) Diapause is determined in the final instar (C 2, D 2). It is likely that the determination takes place shortly after ecdysis (C 3).

(8) Ecdysis to the final instar is postponed by long photoperiods (C 1, D 1) but appears to be stimulated by those of slightly more than equinoctial length (box A).

Experiment 2 (11 February to 24 April 1952)

Objects: To determine the roles of food and temperature in the initiation of metamorphosis in spring.

Material: Final instar: 32 larvae (12 ♂, 20 ♀) collected on 10 February 1952 when the natural photoperiod was 09.40.

Table 4. *Experiment* 2. *Time in days from beginning of experiment* (11 *February*) *to metamorphosis*

Photoperiod	Group		Males	Females
Constant at 06.00	(A) Av. temp. 22° C.; range 21–27° C.	(1) Fed	30, 33, 34	21, 28 (2), 48, 66
		(2) Starved	No response (1 ♂, 3♀)	
	(B) Av. temp. 11° C.; range 10–15° C.	(1) Fed	No response (3 ♂, 5 ♀)	
		(2) Starved	No response (1 ♂, 3 ♀)	
Natural: increasing from 09.40	(C) Av. temp. 15° C.; range 12–18° C.	(1) Fed	27 (2), 42, 64	34, 51, 52, 54

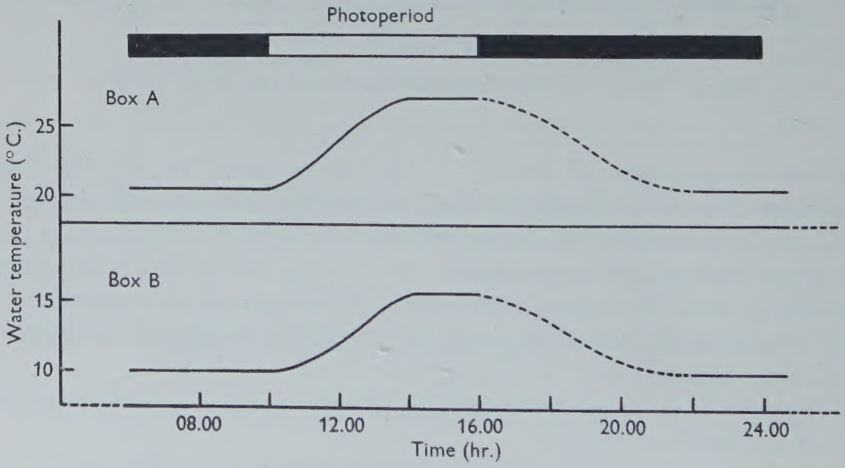

Fig. 4. Exp. 2. Daily temperature fluctuations in relation to photoperiod.

Conditions. Five groups were set up as in Table 4. Groups A and B were illuminated artificially but C was exposed to normal daylight. The amount of food supplied per larva was the same in groups A 1, B 1 and C 1. In all instances in which food was presented it was consumed. Temperature fluctuations in A and B are shown in Fig. 4.

Results. Only groups A1 and C1 proceeded to metamorphosis; other groups showed no response, although the larvae remained alive and active. All larvae in A1 and C1 had embarked on metamorphosis within 66 days of beginning the experiment.

Conclusions. (1) Both food and an average temperature of more than 11° C. are necessary for metamorphosis to proceed.

(2) Absolute photoperiod is not a natural factor necessary for metamorphosis, since in A1 it was well below the lowest value experienced in nature (about 07.48).

(3) By February, diapause development has been completed in nature; thus, in spring, metamorphosis occurs as a simple response to adequate food and temperature.

Experiment 3 (27 October 1952 to 31 May 1953)

Object. To determine whether exposure to low temperatures in the penultimate instar is necessary for diapause to be prevented in the following spring.

Material. Penultimate instar: 7 larvae collected on 27 October 1952.

Conditions. Larvae were kept in a tray by a window at an average temperature of about 18° C.; range 13–23° C. They experienced normal daylight and a natural sequence of photoperiods. They were fed and inspected approximately once a week. Results were not expressed quantitatively.

Results. Two assumptions were made in interpreting the results: (1) larvae in the penultimate instar on 27 October would normally spend the winter in that state, and (2) an adequate period of exposure to low temperature had not already taken place by that date.

Ecdysis to the final instar began between 18 and 25 March 1953. By the end of April all larvae had entered the final instar. All showed signs of metamorphosis before the experiment was concluded at the end of May; thus no larvae entered diapause. This experiment has controls in nature and in Exp. 1, A4. It is noteworthy that ecdysis was restricted to a period shortly after the spring equinox. This provides a comparison for Exp. 1, box A.

Conclusions. (1) Larvae entering the final instar in spring do not require to have experienced winter temperatures in the penultimate instar in order that diapause may be prevented by increasing photoperiods.

(2) It seems likely that ecdysis to the final instar is stimulated by photoperiods of slightly more than equinoctial length (cf. Exp. 1, conclusion (8)).

DISCUSSION

The figures in parentheses refer to the conclusions listed after each experiment in the previous section. The first number indicates the experiment and the second the conclusion.

Induction of diapause. In nature, diapause is restricted to larvae entering the final instar after the end of May. Diapause is determined in the final instar (1:7) and can be induced by two kinds of photoperiodic regime: (i) natural photoperiods decreasing by normal seasonal decrements (1:4), and (ii) constant photoperiods corresponding to 12 May or 31 July (1:5). On the other hand, it can be prevented by an increase in photoperiod, whether this be gradual or sudden (1:6). The size of the daily increments or decrements which can be appreciated is not known, but the induction of diapause after the end of May in nature indicates that increments of less than 2 min. per day cannot prevent diapause. If the same threshold value holds good for decreasing photoperiods, larvae would only begin to discern these after 11 July, when decrements begin to exceed 2 min. per day. This mechanism of diapause determination obviates the necessity for larvae entering the final instar in spring to postpone emergence for 1 year, but, at the same time, it extends the emergence period. The extent to which the emergence period is lengthened will depend upon, and bear an inverse relation to, the value of the threshold increment of daylength that evokes a response.

Ecdysis to the final instar. It appears that ecdysis to the final instar receives especial stimulus from photoperiods of slightly more than equinoctial length (1:8, 3:2). This can be seen as a further mechanism assisting seasonal regulation, since it will tend to restrict the stage when diapause is determined to the season when photoperiodic changes are most pronounced.

Diapause development. Exposure to temperatures averaging 6° C. is not necessary to complete diapause, and insignificant diapause development had occurred after 12 weeks of such treatment (1:2). Since diapause development proceeds slowly at 20–25° C. (1:1), it appears that the optimum temperature for its completion must lie between 6 and 20° C. It is unusual for the optimum temperature for diapause development to lie within the temperature range of normal morphogenesis (Andrewartha, 1952), and thus it is likely that in this case it occurs near the lower end of the range 6–20° C., since the lowest temperature at which metamorphosis can proceed lies between 11 and 15° C. (2:1). It is reasonable to suppose that diapause development may proceed most rapidly at about 10° C. In nature, it seems clear that it must be completed in autumn, before temperatures fall below an average of 6° C. If this were not so, some members of the population would have to fulfil variable periods of diapause development before proceeding to metamorphosis in spring, and the general effect of synchronization would be lost. Most ecdyses to the final instar occur in August and September (1:8 and Corbet, 1956), suggesting that in nature diapause development must be completed in less than 60 days. (Water temperatures between September and November usually lie between 10 and 15° C.) For this to be possible diapause development would have to proceed twice as fast at 10 as at 23° C., but this does not demand a temperature coefficient of an unusual order.

In the light of the above discussion, the following hypothesis may be advanced to account for the completion of diapause development in *A. imperator*: Diapause development has a temperature optimum near 10° C. and, in nature, proceeds at a rate such that it is completed by the end of November. After this date, temperatures fall below a value at which it can proceed. Post-diapause development (metamorphosis) cannot occur below 11° C., but increases in rate as the temperature rises. Thus, diapause development does not proceed at its optimum rate until temperatures fall too low to permit metamorphosis.

Concluding note. Much further work is required to obtain a sound knowledge of the responses involved in the seasonal regulation of *A. imperator*. The experiments described above demonstrate that changing photoperiod is an important agent in determining diapause, but they do not permit firm conclusions to be drawn concerning the effect of temperature on the rate of diapause development. Since, in this case, the rate of diapause development represents the criterion upon which the presence of diapause is judged, it is clearly of the greatest importance that the role of temperature be properly understood. The most urgent steps towards a better understanding of this problem are: (1) the design of experiments in which a constant temperature is maintained independent of photoperiod, and (2) regular autumn collections of wild larvae to determine accurately when diapause development is completed in nature.

SUMMARY

1. Preliminary experiments have been conducted to determine the environmental factors influencing the induction and termination of diapause in the Emperor Dragonfly, *Anax imperator* Leach.

2. A facultative diapause is located in the final larval instar and takes the form of an interruption of morphogenesis such that, at about 23° C., metamorphosis does not occur until some 105 days after ecdysis. In non-diapause larvae this period is about 30 days. Feeding continues during diapause development.

3. Diapause appears to be determined in the first few days of the final instar. It can be induced by decreasing photoperiods, irrespective of the lengths of day involved, and also by a regime of unchanging photoperiods of a length corresponding to 31 July.

4. Diapause can be prevented by exposure to an increase in photoperiod immediately after ecdysis. This may take the form of a single large increase (of more than 20 min.), or of successive small increments such as occur naturally in March and April. It is possible that under unnaturally long photoperiods larvae do not respond normally to regular increments.

5. Ecdysis to the final instar appears to be stimulated by photoperiods of slightly more than equinoctial length. This tends to localize the sensitive stage to seasons when changes in day length are most pronounced.

6. A previous exposure to winter temperatures in the penultimate instar is not necessary for diapause to be averted in spring.

7. It is likely that diapause development proceeds most rapidly at a temperature near 10° C. Negligible diapause development had occured after exposure to 6° C. for 12 weeks, although variation in the period necessary for its completion was then reduced.

8. Diapause development has already been completed in nature by February. After this, metamorphosis occurs as a simple response to adequate temperature and food. It cannot take place when the average temperature is below 11° C. Metamorphosis is not prevented by a regime of decreasing photoperiods.

I should like to express my sincere thanks to Prof. V. B. Wigglesworth, under whose supervision this work was carried out, and to Dr A. D. Lees for helpful advice and criticism. I am grateful to the Department of Scientific and Industrial Research for financial assistance while the work was in progress.

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APPENDIX

(1) *Results of Experiment* 1 (*all times given in days*)

A 1. Dates of ecdyses to final instar and (time elapsing to metamorphosis)
Males: 21/7 (84); 21/7 (133); 23/7 (122); 28/7 (117)
Females: 7/9 (87); 9/9 (125); 16/9 (65); 24/9 (121); 3/10 (86); 24/1 (47)*

A 4. Dates of return to 23° C. (italics), dates of ecdyses to final instar and (time elapsing to metamorphosis)

Period of exposure to 6° C. in penultimate instar	
3 weeks	Males: *11/8*, 4/10 (78); *11/8*, 22/9 (107) Females: *11/8*, 1/10 (99); *11/8*, 28/9 (101)
6 weeks	Males: *1/9*, 11/10 (105) Females: *1/9*, 7/10 (103); *1/9*, 10/10 (89); *9/10*, 16/10 (101)[a]
9 weeks	Males: *22/9*, 20/10 (84); *22/9*, 20/10 (104) Females: *22/9*, 22/10 (88); *30/10*, 19/2 (34)[ab]
12 weeks	Males: *13/10*, 17/2 (42)[b]; *20/11*, 1/12 (61)[a] Females: *13/10*, 30/10 (88); *13/10*, 16/11 (109)

[a] Collected on 25 August 1952.

[b] These larvae experienced increasing photoperiods throughout the final instar and showed no diapause.

* This larva experienced increasing photoperiods throughout the final instar, and has been omitted from the averages in Table 1.

A5. Dates of return to 23° C. (italics) and (time elapsing to metamorphosis)

Period of exposure to 6° C. in final instar	
3 weeks	Males: *26/9* (101); *26/9* (89); *14/10* (95) Females: *21/10* (97)
6 weeks	Males: *11/10* (93) Females: *14/10* (90); *11/11* (95)
9 weeks	Males: *2/11* (93); *7/11* (104) Females: *8/11* (90); *2/12* (94)
12 weeks	Males: *25/11* (82) Females: *17/11* (89); *22/11* (108)
16 weeks	Females: *19/1* (65)*

* This larva experienced increasing photoperiods throughout the final instar.

A6. Time elapsing from beginning of experiment to metamorphosis
Males: 102, 108, 136, 174

A7. Dates of return to 23° C. (italics) and (time elapsing to metamorphosis)

Period of exposure to 6° C. in final instar	
3 weeks	Males: *21/9* (93) Females: *21/9* (75); *21/9* (94)
6 weeks	Males: *9/10* (106); *9/10* (90) Females: *9/10* (75)
9 weeks	Males: *30/10* (122) Females: *30/10* (96); *30/10* (87)
12 weeks	Males: *20/11* (74); *20/11*(93) Females: *20/11* (95)

C1. Dates of ecdyses to final instar, photoperiod at ecdysis and (time elapsing to metamorphosis)

Males: 1/10, 19.51 (48); 7/10, 20.09 (24);* 11/10, 20.21 (57); 13/10, 20.27 (27)*
Females: 24/7, 16.30 (82); 4/10, 20.00 (60); 20/10, 20.48 (32);* 23/10, 20.57 (80)

C2.

Sex	Date of ecdysis	Photoperiods at ecdysis		Increment experienced at transfer	Time from ecdysis to meta-morphosis
		Box A	Box C		
Males	26/7	15.44	16.33	00.49	91
	15/9	12.43	19.03	06.20	74
	19/9*	12.27	19.24	06.57	29
Females	19/8	14.26	17.45	03.19	73
	26/8*	13.59	18.06	04.07	19
	11/9	12.59	19.00	06.01	67

* Non-diapause larvae.

C3. Time from beginning of experiment to metamorphosis:
Males: 54, 78, 87, 108
Females: 78

D1. Time from beginning of experiment to metamorphosis:
Males: 84, 92, 100, 103, 106, 120, 139
Females: 77, 86, 103, 112, 114, 129, 144

D2.

Sex	Date of ecdysis	Photoperiods at ecdysis		Change experienced at transfer	Time from ecdysis to metamorphosis
		Box A	Box C		
Males	24/7	15.51	15.30	−00.21	104
	25/7	15.48	15.30	−00.18	133
	31/7	15.30	15.30	None	117
	25/7	15.48	15.30	−00.18	129
Females	25/7	15.48	15.30	−00.18	135
	3/10*	11.33	15.30	+03.57	27

* This is a non-diapause larva and has been omitted from the averages given in Table 3.

METACHRONISM AND FREQUENCY OF BEAT IN THE PERISTOMIAL CILIA OF *STENTOR*

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INTRODUCTION

Co-ordination between cilia has attracted the attention of a number of people in the past and many theories have been advanced to account for it. A summary of these theories was given by Gray (1928). Verworn suggested that ciliary co-ordination was mechanical, each cilium being mechanically stimulated by its neighbour. This theory was rejected by Parker (1905) because the metachronal wave does not always travel in the same direction as the ciliary beat. Parker suggested that metachronal rhythm was maintained by the passage of excitatory stimuli. Support for this theory was provided by Grave & Schmitt (1925) who found, in the gill epithelium of molluscs, intercellular fibrils which, they claimed, were concerned with ciliary co-ordination. The fibrillar and 'silver-line' systems of ciliate Protozoa are said to have this function; e.g. Taylor (1920) showed that co-ordination in the oral membranellae of *Euplotes* was disturbed by cutting the fibril that runs beneath them.

If the metachronal rhythm of a ciliated epithelium is the result of the passage of excitatory stimuli, then, as Gray (1928) said, it is likely that the co-ordination manifested in metachronal rhythm is independent of mechanical activity. Qualitative evidence for this proposition was produced by Worley (1934*a*, *b*) in lamellibranchs and ciliate Protozoa. He deduced from work on the effects of drugs and anaesthetics that metachronism and beating were separate activities of the ciliary mechanism in these two types of animal.

Quantitative work was carried out by Gray (1930) and Lucas (1932), both of whom used cinematographic methods for the determination of the activity of cilia of lamellibranch gills. Both workers concluded that while the frequency varied with temperature, the metachronal wave-length did not. It follows that the velocity of wave propagation (frequency × wave-length) and the frequency must be proportionately related. Lucas believed, however, that the contractile and co-ordinating mechanisms of the ciliated cell were physiologically distinct functions and that 'each should be able under certain circumstances to act independently of the other'.

Metachronism has attracted much interest, but its relation to the contractile activity of the cilium is still not well defined. The present work was undertaken to discover how far these two factors would behave as independent functions of the ciliary mechanism.

MATERIAL AND METHODS

The animal selected for this work was *Stentor polymorphus* (Ehrbg.). The peristomial cilia of this animal are arranged in a single row which forms an almost complete circle around the broad oral end of the body. The effective stroke of the ciliary beat is directed abaxially and aborally and, in beating, the cilia pass through an angle of about 140° in a plane which is radial to the peristome and in line with the long axis of the body. Metachronal waves pass along the line of cilia at right angles to the plane of beat. The arrangement and metachronal properties of these cilia make them ideal for an investigation of the relations between frequency and metachronal rhythm. No interkinetic periods have been observed in the beating of these cilia as were recorded by Gray (1930) in certain of the cilia of *Mytilus* gill epithelia.

For observation of cilia and measurement of beat frequency, a simple stroboscope was constructed from a small electric motor which was run from a 24 V. direct current supply and controlled by a variable resistance. The central spindle of the motor carried a small mirror and was connected to a revolution counter. Light reflected from a 'Pointolite' source was directed via the rotating mirror on to the substage mirror of the microscope, which was therefore illuminated for only a small fraction of each revolution. When the frequency of rotation of the mirror was the same as the frequency of ciliary beat, then the cilia appeared to remain motionless. The metachronal wave-length was measured from photographs taken at a magnification of ×400 with this stroboscopic illumination.

A constant environment as regards salts and pH is essential. Accordingly, the *Stentor* used were cultured in a medium made up from inorganic salts. Wheat grains and a culture of small protozoans (usually *Chilomonas*) were added to provide food for the *Stentor*, and all experiments were carried out in this culture medium with added drugs, salts or viscous substances as required. The medium contained NaCl (1·4 mM/l.), KCl (0·055 mM/l.), $NaHCO_3$ (0·045 mM/l.), $CaCl_2$ (0·035 mM/l.) and $CaH_4(PO_4)_2 . H_2O$ (0·006 mM/l.), made up in distilled water at about pH 6·8.

EXPERIMENTAL

A. *Temperature and ciliary activity*

(i) *Introduction*

It has been known for about 100 years that temperature influences the rate of ciliary activity. This was demonstrated by Gray (1923), who determined the rate of transport of a small platinum plate over the surface of *Mytilus* gill at different temperatures. This ciliary activity is, however, the resultant of several components which were not treated separately in this earlier work.

Gray (1930), again using *Mytilus*, showed that although the beat frequency changed systematically with change in temperature, the metachronal wave-length did not. Lucas (1932) obtained the same result in work on the lateral gill cilia of *Modiolus*. This result led him to conclude that the rate of propagation of the wave varied 'proportionately' with the frequency.

In the present work frequency and wave-length were measured over the temperature range from below 1° C. to above 35° C. The velocity of propagation of the metachronal wave was calculated from these observations.

(ii) *Experimental*

The animals were placed in a hanging drop in a closed chamber which formed part of the floor of a water-bath in which a constant circulation of water was maintained. The temperature was measured with a thermometer graduated to 0·1° C. which was placed in the water-bath close to the hanging drop. Reductions in temperature were made by addition of ice or cold water to the water in the bath, and the temperature was raised by means of an electric heating coil. A small change in the water-bath temperature as recorded by the thermometer was always accompanied by a corresponding small change in the frequency. *Stentor* will live in a hanging drop of culture medium for over 30 hr. without any appreciable change in beat frequency. It has been assumed that confinement of *Stentor* for 8–10 hr. has no adverse effects on the ciliary activity.

Changes in frequency resulting from changes in temperature are readily reversible within certain temperature limits. Two examples demonstrating this are shown in Fig. 1: (A) following a decrease in temperature, and (B) following an increase in temperature. This reversibility occurs at temperatures down to 0·5° C., but not above 25–26° C. It was found that the frequency had dropped 20% from its original level when an animal was returned to 18° C. after being kept at 28° C. for an hour.

The effect of temperature on frequency, wave-length and wave velocity was investigated in the following manner. The animal was kept at room temperature until the frequency had remained constant for 30 min., and then the temperature was lowered over a period of 30–40 min. to between 0 and 1° C. After the frequency had been steady at this low temperature for 10 min., the temperature was slowly raised over a period of 2–3 hr. to 35–40° C. Sets of readings were taken at intervals throughout the experiment and the experiment was repeated several times. Table 1 shows figures selected from one experiment to show the changes in ciliary activities which occurred when the temperature was raised from 5 to 36° C.

Table 1. *The effect of temperature on frequency, wave-length and wave velocity*

Temperature (° C.)	Frequency (beats/sec.)	Metachronal wave-length (mm.)	Metachronal wave velocity (mm./sec.)
5·3	10·25	0·0275	0·282
10·5	15·6	0·0228	0·355
15·7	22·5	0·0216	0·486
19·9	27·8	0·0204	0·566
25·5	33·0	0·0222	0·733
29·0	36·0	0·0216	0·780
30·6	35·3	0·0216	0·760
36·0	32·8	0·0186	0·610

The cilia continued to beat at temperatures below 0·5° C. At this latter temperature frequencies of about 8 beats/sec. and wave velocities of 150–200μ/sec. were observed. The frequency and wave velocity increased steadily with temperature up to 25–28° C. Above this temperature the rate of increase fell until at 30–32° C. a maximum frequency of 30–45 beats/sec. and a maximum wave velocity of 700–1000μ/sec. were achieved. Higher temperatures caused a decrease from both maxima, while the cilia showed a tendency to beat intermittently. Metachronal rhythm broke down at 36–38° C. and the cilia ceased to beat at a little below 40° C.

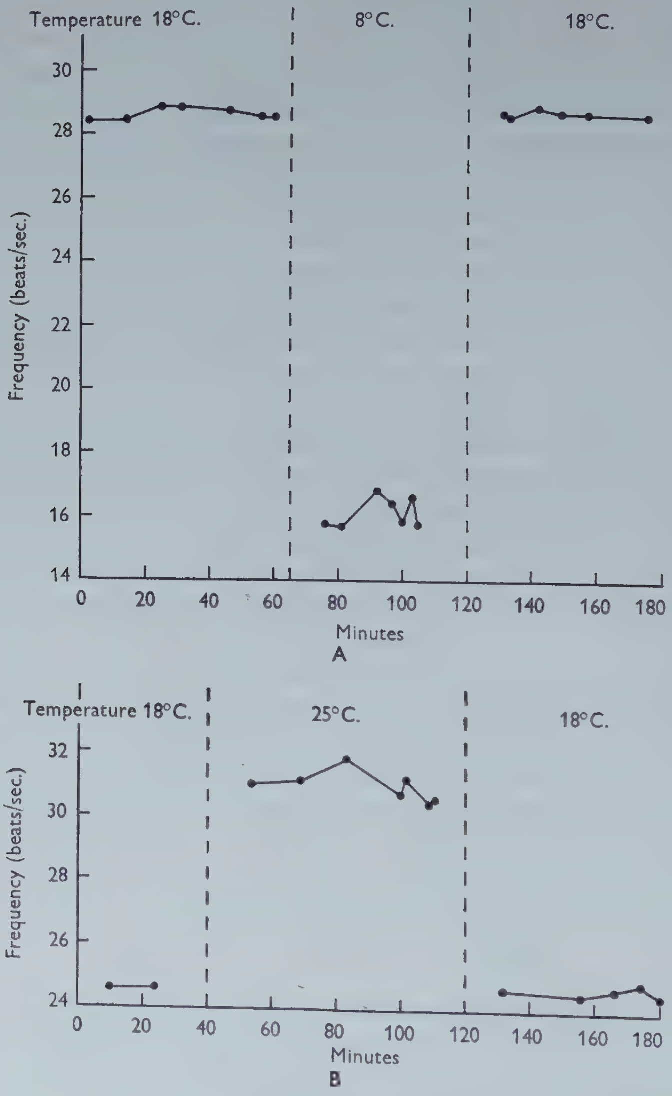

Fig. 1. The reversibility of change of frequency with change of temperature.

Only the effects of temperature on frequency and wave velocity observed in the reversible range from 0 to 25° C. are of importance in this present work.

The empirical equation of Arrhenius expressing the effect of temperature on the velocity of a process affected by temperature may be applied to these results:

$$K = Ce^{-\mu/RT}$$

or

$$\log K = C - \frac{\mu}{RT},$$

where K is the velocity of a process affected by temperature, R, C and μ (the temperature characteristic) are constants, and T is the absolute temperature.

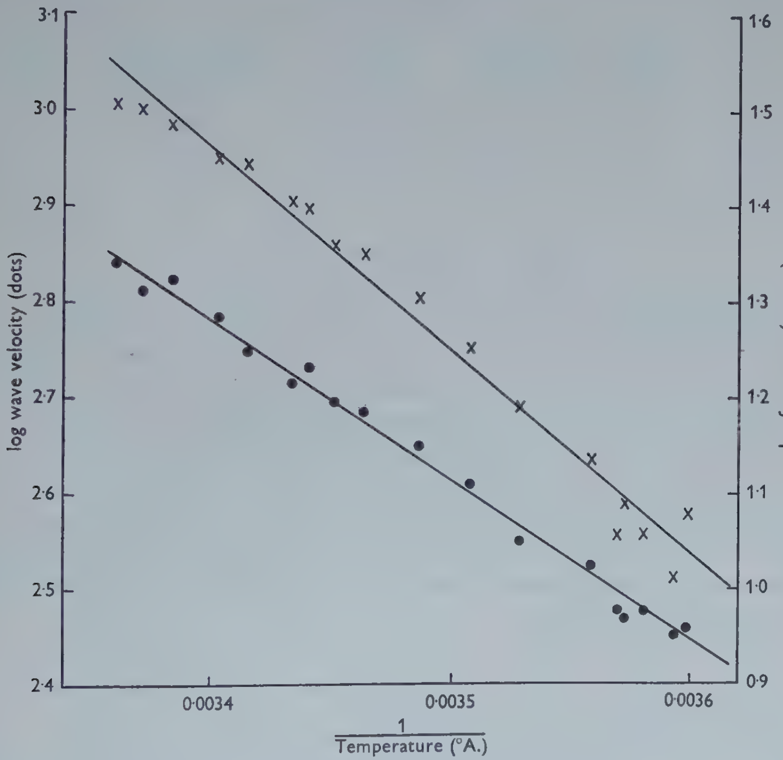

Fig. 2. The effect of temperature on wave velocity and frequency.

Agreement with this equation will be indicated if a straight line is obtained when $\log K$ is plotted against $1/T$.

Fig. 2 shows the result of plotting log frequency and log wave velocity against $1/T$ for a single experiment. Both sets of data fit the calculated regression lines reasonably well, with correlation coefficients of 0·995 for wave velocity and 0·989 for frequency. Estimation of the significance of the difference in slope between the two lines gives a t value of 5·65 with 32 degrees of freedom, which gives a probability of less than 0·001. Thus this difference in slope is significant.

Temperature characteristics were calculated for a series of similar experiments, and a summary of results is given in Table 2. The average of μ for frequency is 11,330 and for wave velocity is 7520; this difference is highly significant.

The findings of these experiments differ from the conclusions of Gray and Lucas in one important respect—the change in wave-length. The wave-length dropped by more than 25% between 5 and 20° C. It is noteworthy that the same trend of average wave-length variations is seen in the results given by Lucas within this temperature range.

Table 2. *The temperature characteristics calculated for frequency and wave velocity*

	Mean μ calories	Standard error of mean	Range of μ calories	Standard deviation of μ values	No. of observations
Frequency	11,330	361	8400–13,800	1590	20
Wave velocity	7520	159	6210–8280	596	14

B. *The effect of other environmental changes*

Experimental method. A similar method was used in all the following experiments investigating the effects of viscosity, $MgCl_2$, $AlCl_3$ and digitoxin on ciliary activity. The animal was kept for some time in the normal medium in order to determine the normal levels of frequency and wave velocity. The medium was then replaced by a test solution of known composition and a number of readings was taken to determine the equilibrium or maximum levels of activity in the test solution. Further sets of readings were taken as the animal was (*a*) returned directly to the normal medium, or (*b*) placed in test solutions of different composition before being returned to the normal medium. The temperature was kept approximately constant throughout the experiments and correction was made for small variations in temperature (not more than 1° C.) which did occur.

(i) *Viscosity and ciliary activity*

There is evidence from the work described above that frequency and wave velocity are separately determined processes. If this is so, then it is probable that these two components of ciliary activity are differently affected by external changes other than temperature. One most important factor is the viscosity of the medium.

Gum arabic has been used to slow down Protozoa to facilitate observation. Experiments with *Stentor* showed that this substance caused permanent change in the ciliary mechanism, an hour's immersion in dilute gum-arabic solution resulting in an irreversible decrease in frequency. It is evidently of no use in this work.

Marsland (1943) recommended methyl cellulose for retarding the movements of *Paramecium*. Dilute solutions of this substance caused completely reversible reductions in the rate of beat of *Stentor* cilia. In the following experiments, therefore, methyl cellulose was dissolved in culture medium to increase the viscosity. The viscosity of test solutions was measured with an Ostwald viscometer.

The changes in frequency which occurred on immersion of *Stentor* in methyl cellulose solutions of several viscosities are shown in Fig. 3, where increase in viscosity is accompanied by a reversible decrease in frequency.

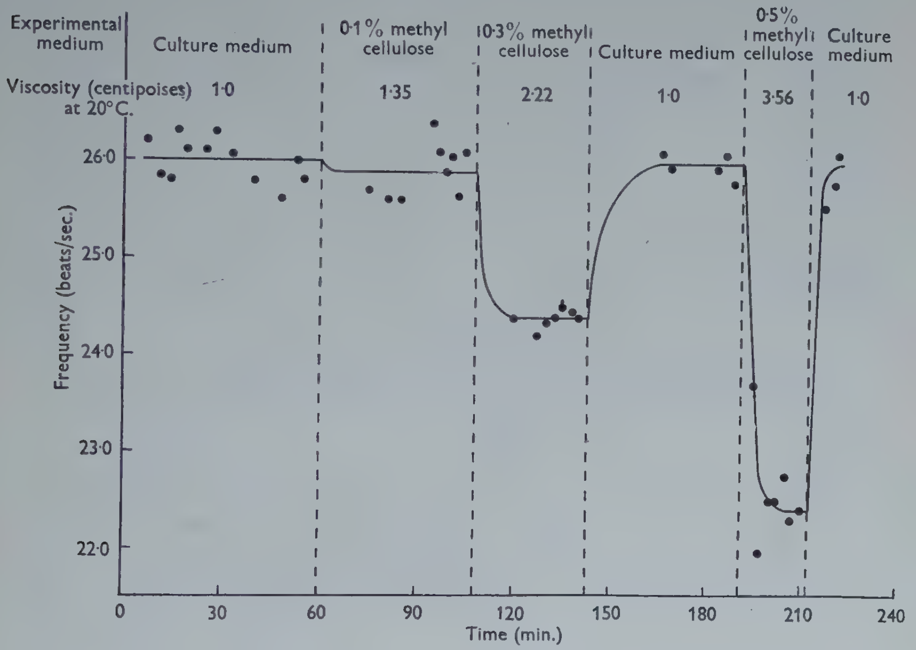

Fig. 3. The effect of successive changes of viscosity on ciliary frequency.

The results of four experiments are shown in a different form in Fig. 4. Increase in viscosity resulted in a proportionate decrease in frequency in each experiment, while the slope of the line was steeper when the normal frequency was high. Frequency decreases of up to 30 % were observed when the viscosity was increased by 250 %.

The results given in Table 3 show that while frequency decreases with increase in viscosity, the wave-length increases, with the result that the changes in wave velocity are all less than 1 %. In all the experiments a similar result was obtained. The mean wave velocity change calculated from fifteen experiments was +0·10 %, with a

Table 3. *The effect of increase of viscosity on frequency, wave-length and wave velocity*

Methyl cellulose concentration (%)	Viscosity (at 20·0° C.) (centipoises)	Frequency (beats/sec.)	Wave-length (mm.)	Wave velocity (mm./sec.)
0	1·00	27·8	0·0216	0·600
c. 0·1	1·35	27·1	0·0224	0·606
c. 0·3	2·22	25·2	0·0239	0·602
c. 0·5	3·56	22·8	0·0261	0·595

standard error of the mean of 0·45 %. Changes in the viscosity of the medium, therefore, do not result in any significant changes in wave velocity.

No change in amplitude was observed in any of these experiments.

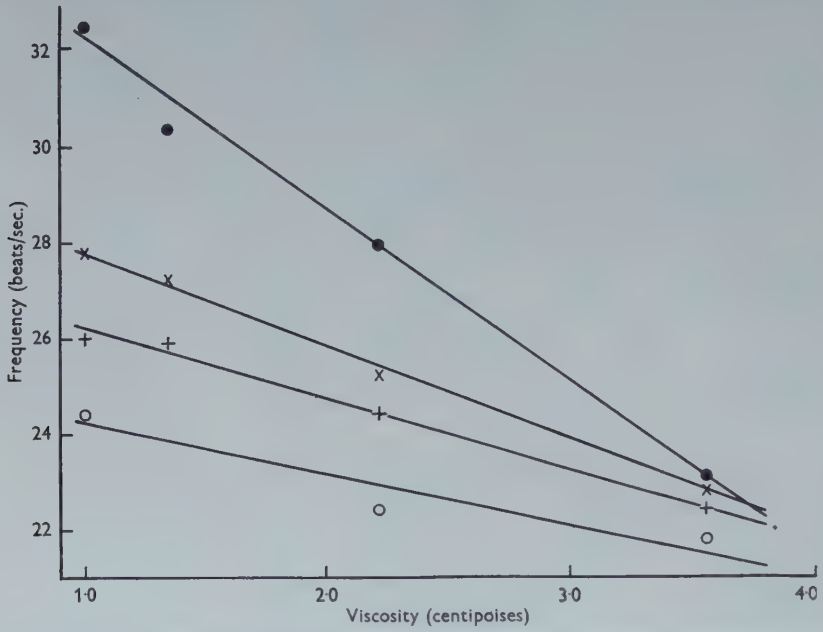

Fig. 4. The effect of viscosity of the medium on frequency; results from four experiments.

(ii) *Magnesium chloride, aluminium chloride and ciliary activity*

The culture medium contains 'Analar' sodium, potassium and calcium salts made up in glass-distilled water. Metallic impurities must therefore be in minute concentration. Magnesium is a metallic ion which, together with the three named above, is considered important in cell equilibria. Gray (1928) stated that the effect of magnesium-free sea water on the lateral gill cilia of *Mytilus* is to increase the rate of beat, and that 'magnesium controls the rate at which they beat by limiting their speed'. There is no magnesium in the *Stentor* culture, so that a more direct approach to the effect of magnesium on ciliary activity should be possible.

Fig. 5 shows the effect on frequency of the addition of $MgCl_2$ to the medium. The change in frequency caused by the addition of $MgCl_2$ was readily reversed on the return of the animal to the normal medium. The increase in frequency varied with $MgCl_2$ concentration as shown in Fig. 6, a maximum of over 20 % increase being reached at about 0·2 mM/l.

At $MgCl_2$ concentrations which increased the frequency, the wave-length was decreased as shown in Table 4, while the wave velocity remained unaltered. The mean wave velocity change calculated from ten experiments was +0·07 %, with a standard error of the mean of 0·49 %. This change is not significant.

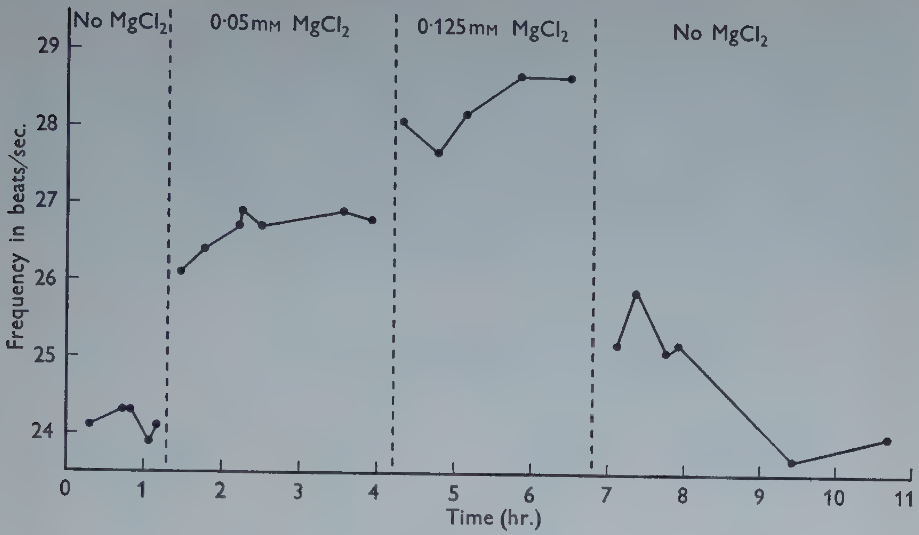

Fig. 5. The effect of two concentrations of MgCl$_2$ on ciliary frequency.

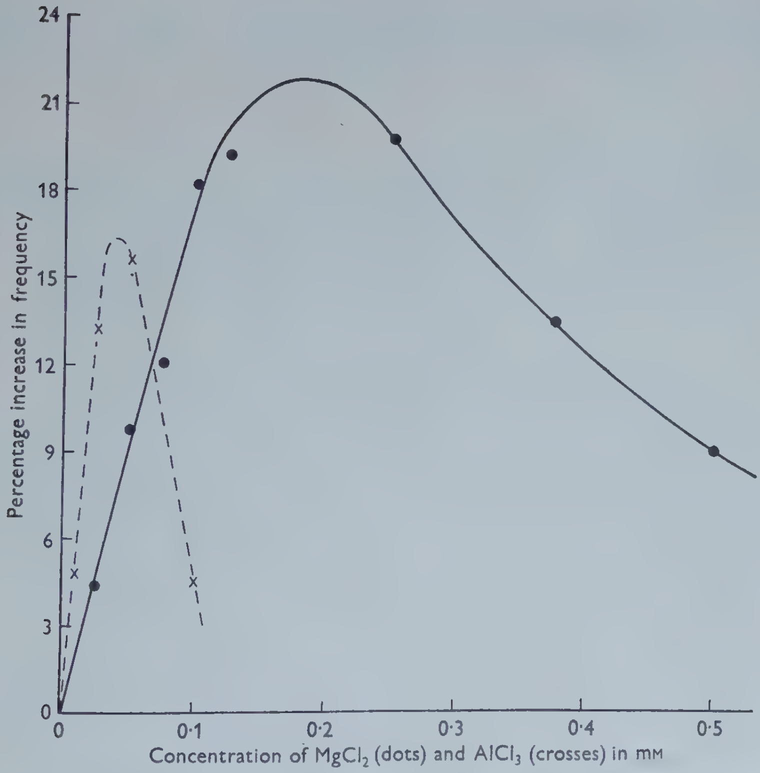

Fig. 6. The effect of MgCl$_2$ concentration and AlCl$_3$ concentration on the frequency.

Heilbrunn (1923) showed that $MgCl_2$ reduced the viscosity of *Stentor* protoplasm, while the chlorides of the monovalent cations K^+ and Na^+ increased protoplasmic viscosity. He attributed this to the effect of these ions on the ionization of protoplasmic colloids. If this is correct, then a trivalent ion might be expected to reduce protoplasmic viscosity even more than a divalent one. Accordingly, experiments were carried out on the effect of various concentrations of aluminium chloride on ciliary frequency. The results of these experiments are shown in Fig. 6. $AlCl_3$ is several times more active in increasing frequency than $MgCl_2$, but this activity falls away sharply at higher concentrations, probably as a result of high toxicity.

Table 4. *The effects of magnesium chloride solutions of two concentrations on frequency, wave-length and wave velocity*

Magnesium chloride concentration (millimolar)	Frequency (beats/sec.)	Wave-length (mm.)	Wave velocity (mm./sec.)
0	24·1	0·0244	0·588
0·050	26·8	0·0222	0·594
0·125	28·6	0·0207	0·591

$MgCl_2$ and $AlCl_3$, which are thought to decrease the viscous resistance of protoplasm, were found to affect frequency and not wave velocity. Thus these two processes are again shown to have different properties.

(iii) *Digitoxin and ciliary activity*

Both the rate of beat of cilia and the rate of conduction of the metachronal wave are altered by change in temperature. Viscous and ionic changes in the medium affect the rate of beat, but not the wave velocity. It would be interesting if change in wave velocity without alteration of frequency could be demonstrated.

Gray (1923) wrote: 'It is, therefore, possible to conceive that motile mechanisms of ciliated cells and of cardiac muscle may be found to have much in common.' The velocity of conduction of the contraction wave in heart muscle (10–40 mm./sec. in tubular hearts—Clark (1927)) lies between the rate of wave propagation in ciliated tissues (0·5–1·0 mm./sec.) and the rate of conduction in nerve fibres (50–1000 mm./sec. in small non-medullated fibres—Prosser (1950)). Substances known to affect the heart, for example digitoxin, may also affect ciliated tissues. Accordingly, the effect of digitoxin on *Stentor* cilia was investigated.

Fig. 7 shows the effects on frequency and wave velocity of a solution containing 0·2 mg./l. of digitoxin. The wave velocity in this example increased by up to 33 %, while the frequency increases were less than 5 %. Changes of a similar order occurred at different digitoxin concentrations as shown in Fig. 8, where each point is the mean of at least two experiments. Since the effect of digitoxin varies with time after application, the results are described in terms of the maximum wave velocity attained, and the frequency at that time.

The greatest wave velocity increase of some 35 % was obtained at a digitoxin concentration of 0·4 mg./l. (1×10^{-6} M), with smaller increases at higher and lower concentrations. The frequency changes followed a different pattern. There was no sharp peak in the graph, but only a steady rise to the greatest increase of 6 % at the highest concentration used.

The results of these experiments show quite clearly that the effect of digitoxin on frequency is qualitatively as well as quantitatively different from that on wave velocity.

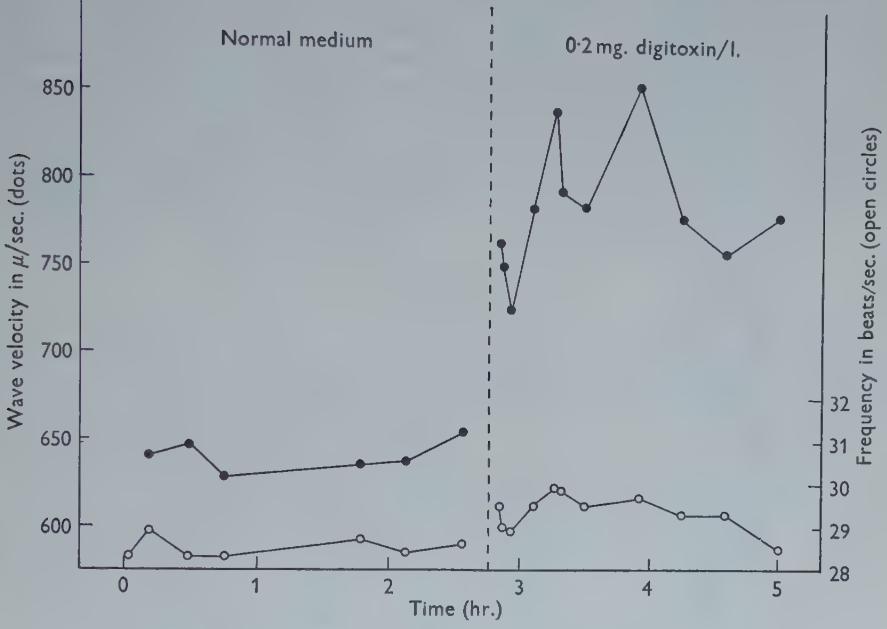

Fig. 7. The effect of digitoxin (0·2 mg./l.) on frequency and wave velocity.

DISCUSSION

In the normal working of a system of cilia such as the present, the pattern of ciliary beat may vary in (1) frequency, (2) amplitude, (3) metachronal wave-length and (4) metachronal wave velocity. The problem is to decide which if any of these is an independent variable of the system. No variations in amplitude have been observed with any of the treatments employed; refinements of experimental technique would be required for measurements of small changes in amplitude. Consideration of amplitude will therefore be omitted. The three remaining variables are mutually related in the equation *wave velocity = frequency* × *wave-length*; so that if any two of them are fixed, the third is necessarily determined.

The experiments on effect of change in viscosity of the medium and of the presence of Mg^{2+} showed that while the frequency and wave-length changed by as much as 30 %, the wave velocity was constant. This indicates that the wave velocity is likely to be a fundamental characteristic of the system. It can be visualized as a process analogous to the rate of conduction of a nerve impulse, probably having a similar system of successive excitation. The rate here is, however, very much slower than in nerve, and it would be unwise to push this analogy too far.

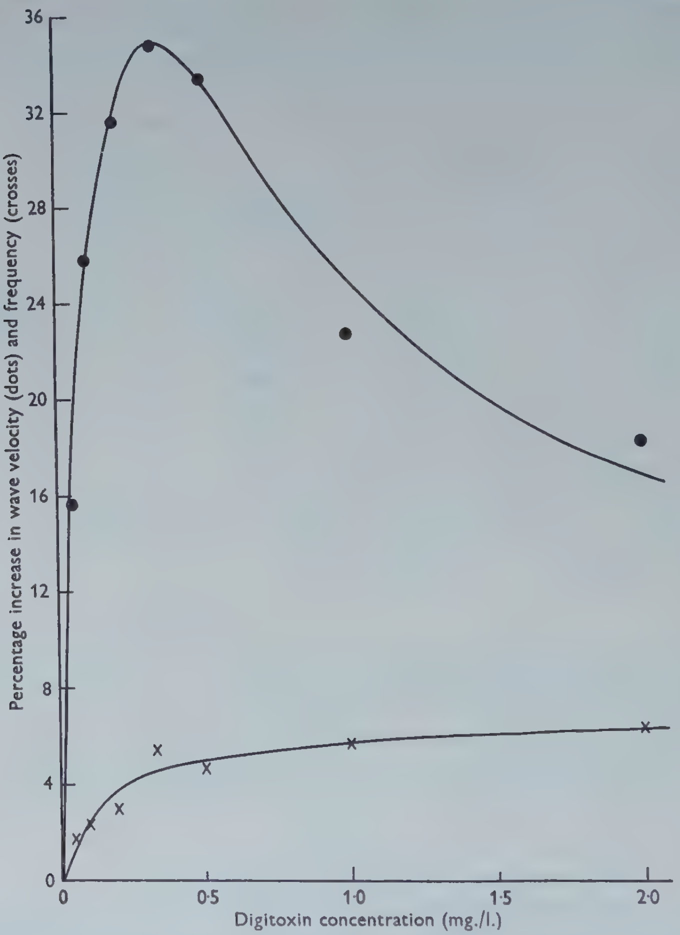

Fig. 8. The effect of digitoxin concentration on frequency and wave velocity.

It is more difficult to decide which of the other two variables is more fundamental. The wave-length determines the angular phase difference between successive cilia, and may be thought of (in Verworn's theory or as suggested by Gray (1930)) as determining a mechanical trigger which acts as a stimulus for the contraction of the next cilium in the succession. But if the wave-length is a fundamental variable, it is difficult to understand why the phase difference should be greatly decreased (e.g. in the digitoxin experiments), when the frequency is only slightly increased and the viscous mechanical conditions remain unchanged. If the wave velocity means anything, it must represent some excitation process, and it is unlikely that there should be two separate methods of excitation of cilia—one neuroid in Parker's sense and the other mechanical—which both serve the same function simultaneously.

The second independent variable is therefore more likely to be the frequency, and the results from digitoxin experiments support this idea. Frequency will clearly be determined by the angular velocity of the cilium, which in turn will be a function of the contractile force exerted and the viscous resistance, both external and internal, encountered by the cilium during its motion. This is consistent with results obtained in experiments in which the external viscosity is increased by addition of methyl cellulose, or the internal resistance is reduced by Mg^{2+} or Al^{3+}.

At the end of each cycle of activity of an individual cilium a new cycle must commence and during this cycle some excitation process must be involved. It is not surprising, therefore, to find that while the frequency can be altered without change in wave velocity, a change in wave velocity (e.g. with digitoxin) is accompanied by a change in frequency. It is perhaps in the relationship between frequency and wave velocity under various conditions that the clue to further investigations of the 'pacemaker' and excitation mechanisms of cilia will be found.

SUMMARY

1. A stroboscopic method was used to determine the frequency of beat of the peristomial cilia of *Stentor polymorphus* (Ehrbg.). The metachronal wave-length was measured from photographs. These two values were used to calculate the rate of propagation of the metachronal wave.

2. The Arrhenius temperature characteristics for frequency and for wave velocity were calculated for the temperature range from 0 to 25° C. The average values found, 11,350 and 7520, were significantly different for the two factors.

3. An increase in the viscosity of the medium by addition of methyl cellulose caused a decrease in frequency, but no significant change in wave velocity.

4. The presence of magnesium chloride in low concentrations caused an increase in frequency, but no significant change in wave velocity. Aluminium chloride caused similar increases in frequency at lower concentrations than magnesium chloride.

5. The drug digitoxin had different quantitative and qualitative effects on the frequency and on the wave velocity. Small increases in frequency occurred over the concentration range in which large increases in wave velocity were recorded. The wave velocity increase reached a maximum at about 0·4 mg./l. of digitoxin and was some six times as great as the frequency increase at this concentration.

6. It is concluded that the mechanical process involved in ciliary activity functions independently of the co-ordination process. A combination of these two factors determines the magnitude of the metachronal wave-length.

The present problem was suggested to me by my supervisor, Prof. J. E. Harris, to whom I should like to express my sincere gratitude for many stimulating discussions, and for his criticisms of the manuscript of this paper. The work was carried out during the tenure of a grant from the Department of Scientific and Industrial Research.

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THE WATER RELATIONS OF EARTHWORMS

II. RESISTANCE TO DESICCATION AND IMMERSION, AND BEHAVIOUR WHEN SUBMERGED AND WHEN ALLOWED A CHOICE OF ENVIRONMENT

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I. INTRODUCTION

In this paper the reactions of whole worms of six species to various water conditions are described, and an attempt is made to correlate the information obtained with the ecological distribution of the species.

II. ECOLOGY OF THE SPECIES USED

Allolobophora caliginosa Savigny, 1826

A. caliginosa is found particularly in cultivated soil, its optimal conditions being in pastures, meadows, and foliated forests (Černosvitov, MS.; Kollmannsperger 1936; Černosvitov & Evans, 1947; Guild, 1948). It has been recorded from clay soil, from humid soil on river banks, under stones and from purely limnic localities (Friend, 1910; Černosvitov & Evans, 1947; Černosvitov, MS.), but is more numerous on light loams than elsewhere, and is absent from very acid peaty soil (Guild, 1948, 1951).

Allolobophora chlorotica Savigny, 1826

This species is found in a great variety of habitats. Although it is a hydrophilous species it can withstand considerable fluctuations in humidity. *A. chlorotica* is frequently found by ponds, lakes and rivers, and less frequently in streams and in subterranean water (Friend, 1891, 1897, 1909, 1911, 1926; Davies, 1950, and personal communication). It is also found in Lake Windermere up to 20 m. from the shore at a depth of about 2 m. (Černosvitov, 1945). In addition, this species also occurs in semi-fixed dunes in Anglesey, where the water table falls to at least a metre during summer (Dr T. B. Reynoldson, personal communication).

Although never in great numbers, *A. chlorotica* is frequently found in gardens, arable fields and meadows, especially amongst the roots of plants (Evans & Guild, 1947). Populations are greatest on alluvium, clay and gravelly sand (Kollmannsperger, 1936; Guild, 1948, 1951).

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Allolobophora terrestris Savigny, 1826 forma *longa* Ude, 1885

A. terrestris f. *longa* is one of the commonest British earthworms. It is a typical pasture form, and is found in cultivated soils. Although it is a terrestrial animal, it is occasionally found in limnic localities, and is particularly abundant in the moist soil on the banks of rivers and lakes (Černosvitov and Evans, 1947; Guild, 1948).

Dendrobaena subrubicunda Eisen, 1874

This species is found in places rich in decaying organic matter: in compost heaps, leaf-mould, and in rich garden soil (Friend, 1910, 1910–11, 1911, 1926; Černosvitov, MS.; Černosvitov & Evans, 1947). Although *D. subrubicunda* should be regarded as a terrestrial species it is occasionally found in limnic habitats.

Lumbricus rubellus Hoffmeister, 1843

This species is a stenotope humus form requiring a certain humidity, and is therefore absent from very moist localities and dry steppes (Kollmannsperger, 1934, 1936). It occurs in places rich in humus, under moss, gardens, pastures and river banks (Friend, 1910, 1910–11, 1926; Moszyński, 1928; Černosvitov & Evans, 1947). It is most numerous on light loams and is tolerant of acid soils, where it often becomes the dominant species (Guild, 1948, 1951). It is rarely amphibious (Černosvitov & Evans, 1947).

Lumbricus terrestris Linnaeus, 1785

L. terrestris is a purely terrestrial, deeply burrowing species. It is tolerant of a wide range of pasture types, altitude and soil acidity. According to Černosvitov & Evans (1947) *L. terrestris* shows a preference for clayey soil, but Guild (1948) found that it is most numerous on light loam.

III. THE RESISTANCE OF EARTHWORMS TO DESICCATION

Water rapidly evaporates from earthworms. In natural environments fatal desiccation is avoided either by diapause or by a retreat to the subsoil. Some species undergo a facultative diapause, rolling up into a tight ball, and remaining inactive in a spherical earthen cell, when conditions become too dry or otherwise unfavourable. Others have an obligatory diapause which is not dependent on external factors, but which occurs during the summer when adverse conditions are most likely to prevail. Still others retreat to the subsoil. Nevertheless, earthworms can withstand a considerable degree of desiccation (Schmidt, 1918; Hall, 1922; Jackson, 1926).

In my experiments *Lumbricus terrestris* and *Allolobophora chlorotica* were used. The water content of each species was found so that the effect of water loss on the concentration of the coelomic fluid could be estimated.

(1) *Water content*

The worms were kept on moist filter-paper for 3 or 4 days before the experiments, to clear their guts of earth. They were slit open on filter-paper and dehydrated individually in a vacuum desiccator at 100° C. with phosphorus pentoxide as the dehydrating agent. The water content of the filter-paper was found in the same way, and the results for the water content of the worms were corrected accordingly.

A period of 24–29 hr. was necessary for constant weight to be reached. Determinations were made for seven *L. terrestris* (fresh weights from 3·230 to 6·240 g.) and six *A. chlorotica* (fresh weights from 0·261 to 0·363 g.). The water contents, expressed as a percentage of the body weight, were: *L. terrestris* from 81·9 to 87·7, mean 84·8 (S.D. 1·597); *A. chlorotica* from 74·7 to 82·2, mean 80 (S.D. 2·4860). This difference between the means is highly significant. Application of the '*t*' test shows that there is a probability of only 1 in 500 of this difference occurring by chance.

(2) *Survival after partial desiccation*

Again, the worms were kept on filter-paper before the experiments to clear the gut of earth. They were weighed individually and then covered with wire gauze or nylon net and placed in desiccators with anhydrous calcium chloride as the dehydrating agent. After desiccation to various degrees they were re-weighed and placed on moist filter-paper and left to recover. The worms were deemed to have recovered if they lived for at least 2 days after the experiment.

About 20–24 hr. were required for *L. terrestris* to lose 50 % of its body weight, and 3 hr. for the same loss in *A. chlorotica*. The temperature ranged between 15 and 18° C. during the experiments.

The results are shown in Table 1. The greatest loss which *L. terrestris* can survive is about 60 % of body weight, i.e. 70 % of the water content of the body. Several of the *A. chlorotica* made a temporary recovery, i.e. they regained the power of movement but died within a day. The limit for true recovery in this species is about 60 % loss of body weight, which is 75 % of the water content of the body.

My results differ from those obtained by Jackson (1926) for *L. terrestris*, and by Hall (1922) for *A. chlorotica*. In Jackson's experiments the limit of tolerance of loss of body weight was between 43 and 50 % as compared with 57–59·7 % in mine, the time taken for desiccation being very much shorter, 5–9 hr. instead of more than 20 hr., which may be responsible for the different results.

Hall found that one specimen of *A. chlorotica* survived after a loss of 69·6 % of its body weight, and that all worms desiccated until up to 26 % of their body weight had been lost survived. Losses of more than 26 % were followed by varying degrees of survival. The limit in my experiments (between 57·9 and 60 %) represents a greater loss than in Hall's experiments unless his exception is taken into account. In Hall's experiments desiccation was carried out at 27–30° C. Although no information is available for *A. chlorotica* Wolf (1938*b*, 1941) found the heat-death temperature for

L. terrestris to be 28° C. (exposure time 500 min.), and it seems probable that the high temperatures affected Hall's results.

Table 1. *Recovery of earthworms after desiccation*

No.	% body wt. lost	Recovery	No.	% body wt. lost	Recovery
		L. terrestris			
1	30·3	+	21	55·0	+
2	31·1	+	22	56·4	+
3	34·2	+	23	57·0	+
4	36·7	+	24	59·7	−
5	38·3	+	25	59·8	−
6	39·6	+	26	59·9	−
7	40·4	+	27	60·6	+
8	40·6	+	28	60·8	−
9	42·1	+	29	63·1	−
10	43·1	−	30	63·9	−
11	45·1	+	31	64·0	−
12	47·3	+	32	66·2	−
13	47·5	+	33	68·4	−
14	48·4	+	34	70·2	−
15	48·5	+	35	71·6	−
16	48·7	+	36	72·4	−
17	50·8	+	37	74·4	−
18	52·4	+	38	83·0	−
19	53·5	+	39	85·0	−
20	54·7	−			
		A. chlorotica			
1	28·0	+	12	49·6	+
2	31·0	+	13	52·5	+
3	32·4	+	14	56·2	Temporary
4	34·2	+	15	56·8	+
5	42·2	+	16	57·6	+
6	44·1	+	17	57·9	+
7	44·8	+	18	60·0	Temporary
8	45·3	−	19	61·0	Temporary
9	45·7	+	20	69·2	−
10	45·9	Temporary	21	69·2	−
11	49·0	Temporary	22	72·5	−

+ = full recovery, − = no recovery.

IV. THE RESISTANCE OF EARTHWORMS TO IMMERSION

The ability of earthworms to survive in water has long been known. In 1874 Perrier reported the survival of *L. terrestris* in frequently changed tap water for more than 4 months. Since then similar results have been reported for several species (Colosi, 1925; Nagano, 1934). I made a comparative study of the resistance of six species to immersion.

(1) *Length of survival in soil under water*

A layer of soil 1–1½ in. deep was placed in tanks and covered with 3–4 in. of tap water, care being taken to ensure that no air was left in the soil. Woodland soil was used for *L. rubellus*, and clayey loam for the other species. The water was aerated, and the level maintained by the addition of glass-distilled water. One or two worms were placed in each tank. All species, except *A. caliginosa*, were able to live for long periods under these conditions (see Table 2). As only two specimens of *A. caliginosa* were used, and as there appears to be an initial danger period to be survived (Maluf, 1939, and results for other species), the results for *A. caliginosa* cannot be regarded as conclusive. It will be noted that in all cases some part of the worm's body was normally above the surface of the soil. Only during a spell of very cold weather when the temperature fell to 9° C. did the worms go completely below the soil surface. Colosi (1925) observed that *A. caliginosa* and *Dendrobaena subrubicunda* lived for nearly 8 months under similar conditions, and that the anterior end was kept in the soil, leaving the posterior part in the water. The significance of this is at present unknown, but it may be necessary for respiration. According to Merker (1926, 1928), water filtered through soil loses much of its oxygen. It is possible that in the tanks the soil remained deficient in oxygen despite aeration of the water above.

Table 2. *The survival of earthworms in soil under water*

Species	Soil	Worm no.	Length of survival	Notes on behaviour	Temperature range (° C.)
A. caliginosa	Clayey loam	1	<7 days	Lay on soil surface	20
		2	<7 days		
A. chlorotica	Clayey loam	1	7 days	Anterior or posterior end on surface or whole worm under surface litter	20
		2	31 weeks		9–18
		3	48 weeks		9–20
A. terrestris f. *longa*	Clayey loam	1	14 days	Part of body always above soil surface	18–20
		2	50 weeks		8·5–20
D. subrubicunda	Clayey loam	1	50 weeks	Just beneath soil surface horizontally with anterior ends projecting	9–20
		2	50 weeks		
L. rubellus	Woodland	1	1½ days	Whole worm quiescent on surface.	18
		2	39 weeks	One end on soil surface	9–19·5

(2) *Length of survival immersed in aerated tap water*

In this experiment the tanks each contained five or six worms, and were kept under similar conditions. Unfortunately the tanks were not uniform, which, together with the different sizes of the worms, meant that the weight of worm per standard volume of water and the surface area of the water was not the same in each case. As, however, the water was well aerated these discrepancies were probably not significant.

The results are seen in Table 3. All species are capable of living for many weeks in aerated water without food, and no significant difference is apparent between them. The length of survival may depend on ability to survive starvation rather than submergence.

Table 3. *Length of survival in aerated tap water*

Species	No. of worms	Survival times in days			Volume of water (ml.)	Surface area of water (cm.^{2})	Gram worm/ 1000 ml. water	Temperature range (° C.)
		Min.	Mean	Max.				
A. caliginosa	5	100	100	100	1000	141·1	2·57	11–19
A. chlorotica	6	134	137	141	1232	154	1·51	8–17·5
A. terrestris f. *longa*	6	93	99	124	2304	256	6·51	8–17·5
D. subrubicunda	5	63	72	77	1000	141·1	0·61	11–19
L. rubellus	6	3	78	195	1425	150	3·20	8–17·5
L. terrestris	6	88	137	166	2262	282·8	9·39	8–17·5
L. terrestris	5	35	92	111	2262	282·8	7·83	8–17·5

V. THE BEHAVIOUR OF EARTHWORMS WHEN SUBMERGED

Observations on the activity of submerged earthworms were made, to find out how far the worms can be regarded as viable fresh-water animals under natural conditions. Particular attention was paid to *A. chlorotica* because of its frequent occurrence in limnic habitats.

If specimens of *A. chlorotica* collected from a garden are able to feed and reproduce under water the occurrence of this species in Lake Windermere can be explained by the accidental introduction of worms or cocoons into the lake, and it is then unnecessary to postulate that the Lake Windermere individuals constitute a separate race.

Worms in glass tubes

Straight pieces of glass tubing of a diameter roughly equal to that of the worm were placed in the tanks containing some of the worms living in aerated tap water. The dimensions of the tubes were as follows:

Length	Diameter	Species
18 cm.	1 cm.	*L. terrestris* (tank with 5 worms)
11·5 cm.	6 mm.	*L. rubellus*
9 cm.	5 mm.	*A. chlorotica*

One tube per worm.

All *L. terrestris* specimens entered the tubes during the night, and then spent most of their time in them with either the anterior or posterior end, or a part of both projecting. At intervals, periods of a few days were spent out of the tubes. *L. rubellus* did not usually use the tubes, although occasionally one was found inside. *A. chlorotica* used the tubes but often, as the diameter of the tubes was too great, two folded worms shared a short length. They were transferred to a larger tank and given tubes 12 cm. long and 2 mm. in diameter. They entered these without folding, but kept either the posterior or anterior end projecting.

Worms in soil

Vivaria made to the design described by Wells (1949) were used in these and some following experiments. Each vivarium consisted of two pieces of glass separated by a strip of rubber coated with vacuum grease and kept together by means of six 'G' clamps or 'bulldog' clips (see fig. 2). The size of the glass and the cross-section of the rubber strip was varied.

A single specimen of *A. chlorotica* was added to a small vivarium of $3\frac{1}{4} \times 4\frac{1}{4}$ in. glass plates and with rubber strips $\frac{1}{8}$ sq.in. in cross-section, set up with a layer of clay soil covered with water. This was repeated three times. In each case the worm burrowed straight down, and during the night constructed a U-shaped burrow, from one arm of which the posterior end was projected. There was a tendency for the arm of the burrow housing the posterior end of the worm to be constructed immediately below the aeration jet; when the latter was moved, so the worm moved.

Specimens of *A. chlorotica* from Lake Windermere in a large tank of water-covered soil with plants were observed to burrow incompletely into the soil so that the posterior end projected in the same way.

When placed in a similar vivarium *L. rubellus* showed a different type of behaviour. It did not burrow into the soil, but contrived to keep as much of its body as possible suspended between the glass plates in the small air space above the water.

Like *L. rubellus*, *L. terrestris* did not construct burrows when placed in a larger vivarium (15 × 15 in. plates and $\frac{3}{8}$ sq.in. cross-section rubber), with all the soil below water. One of the three worms used burrowed extensively during the first night, but thereafter remained on the surface of the soil. One of the other two worms burrowed when first placed in the vivarium, but returned to the surface again within 15 min. and made no further attempts to burrow. Numerous attempts to climb out of the vivarium were made by all the worms, particularly at first.

Thus *A. chlorotica* was the only one of the three species which was observed to construct a U-shaped burrow when the earth was covered by water.

Irrigation of the burrow by Allolobophora chlorotica

It was thought possible that *A. chlorotica* might irrigate its burrow (or glass tube) as do many truly aquatic annelids. The worms were watched carefully but no movements could be detected, even when the tank was placed on graph paper. The use of a carmine suspension did not indicate any movement when the worms were in glass tubes or natural burrows. It is possible, however, that such movements only come into play when the water lacks oxygen, or has excess carbon dioxide, or when there is a combination of both of these factors. To test this possibility each of the conditions was produced experimentally.

A high concentration of carbon dioxide caused the worms to withdraw completely into their tubes or burrows, and to remain quiescent. This occurred when the whole of the water was replaced by carbon dioxide saturated water, or when a little was introduced in the region of the exposed anterior or posterior end. A slight increase in the carbon dioxide content of the water produced no response.

When the tap water was replaced by cooled boiled water (oxygen lack) the worms either left their tubes or showed no response.

A combination of oxygen lack and carbon dioxide excess, produced by bubbling carbon dioxide for 15 min. through cooled boiled water, covered with a layer of liquid paraffin, did not elicit a clearly marked response. Complete replacement of the original tap water by water prepared as above resulted in the worms withdrawing their exposed ends for a few minutes. A little of the water, introduced in the region of the exposed parts of the worms, produced no response. Nor could any currents be detected by carmine suspension.

Thus no circulation of water through the tube occurs under any of the above circumstances.

Feeding

Five *A. chlorotica* specimens were placed in aerated tap water to allow the gut to become empty. They were examined at intervals, but even after a month the gut still retained some earth. In two worms the whole of the anterior part of the gut as far back as the clitellum was quite empty, while the rest of the intestine contained a small amount of earth throughout its length. These were placed in a tank with soil, leaf-mould, and bits of shredded cabbage leaf and carrot, and covered with water 3–4 in. deep. Although the worms did not diminish in size as those left in water without food, no positive evidence of feeding could be obtained, the gut seeming to be in exactly the same condition at each examination, even after 4 months. Indirect evidence for feeding is that worms in water with some soil survive for much longer periods than those without soil. (See also evidence given in the paragraph on the development of cocoons, below.)

Cocoon production

To see if worms can reproduce when submerged, six mature worms were placed in a tank containing water and some autoclaved soil. Food was washed before adding it to the tank to avoid the accidental introduction of cocoons. No reproduction was observed during a year.

Development of cocoons

Eighteen cocoons of *A. chlorotica*, collected from culture pots, were placed in a dish of aerated tap water.

All but three cocoons produced young worms, one producing two. On hatching the young worms were transferred to a shallow dish containing tap water, some soil and small pieces of organic matter. These worms produced castings and one was observed making a cast. The alimentary canal, empty on hatching, soon became full of earth. Three worms were weighed on hatching, and placed in a dish with water and soil. They were then weighed at intervals. The results, which are given in

Fig. 1, show conclusively that the worms grow under these conditions, and therefore are able to feed under water.

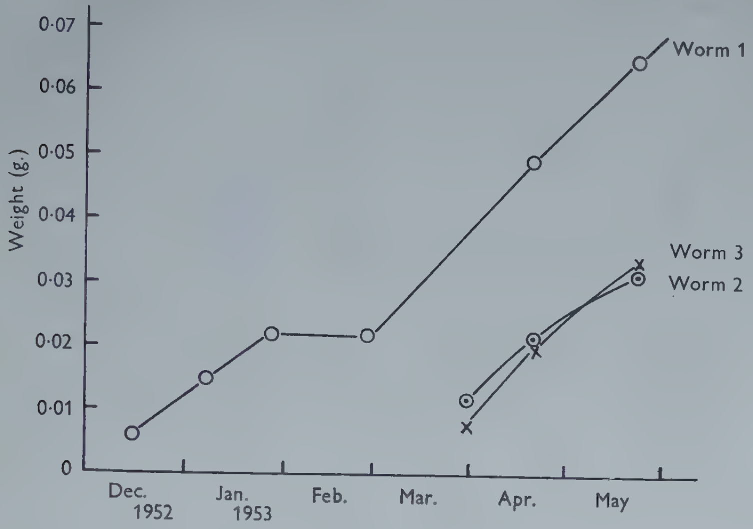

Fig. 1. Increase in weight of young *A. chlorotica*.

VI. THE BEHAVIOUR OF EARTHWORMS WHEN ALLOWED A CHOICE OF ENVIRONMENT

All the species investigated are able to live in water when forced to do so, but only some of them occur in water under natural conditions. The following experiments were designed to study behaviour as a possible cause of differences in distribution.

Worms in soil

These experiments were made in glass-sided vivaria as described above. Some tap water was put into the apparatus, and soil was added. The amounts of soil and water were adjusted so that the final result was water-saturated air-free soil under aerated water on one side, and water-saturated air-free soil topped by moist air-containing soil on the other (Fig. 2). The positions of the worms were recorded daily by drawings. They moved little during the day, so only one observation every 24 hr. was made.

The following factors were varied during the experiments:

(*a*) *The distance between the glass plates.* This should be wide enough to allow the worms to move freely, and narrow enough to ensure that they can be seen. The finding of *A. chlorotica* was particularly difficult owing to their small size and grey colour, and in several of the experiments on this species many of the worms escaped observation.

(*b*) *The nature of the soil.* This was varied because chemical factors might alter the results. The following soils were used, giving a wide variation in organic content: leaf-mould, clay, equal parts by volume of leaf-mould and clay, and washed fine silver sand. The leaf-mould was soil enriched by decayed oak and hawthorn leaves.

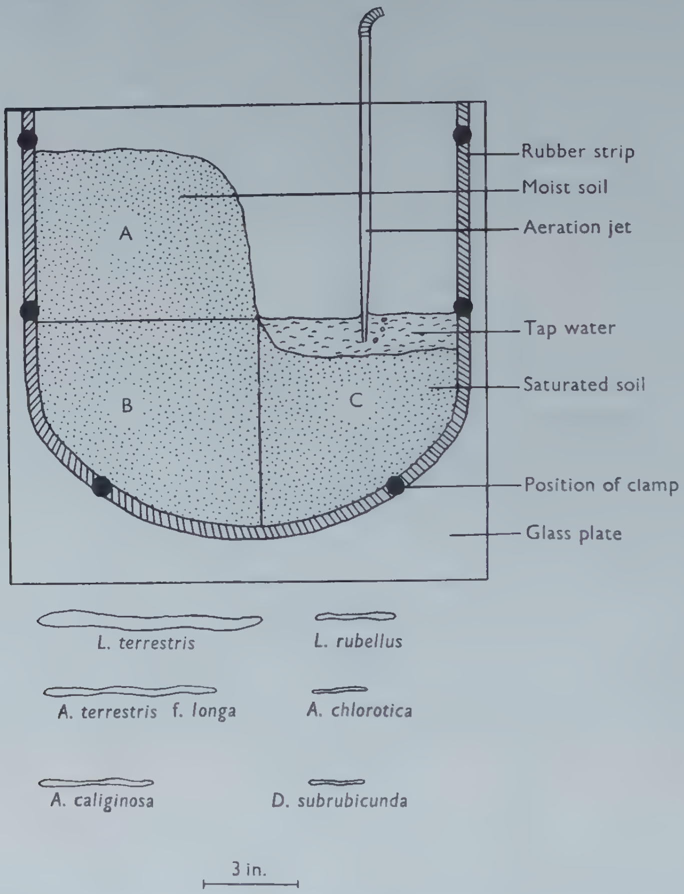

Fig. 2. Diagram of glass-sided vivarium showing the size of the worms relative to the apparatus.

The results are shown in Table 4. For the purpose of making the analysis shown in the table the soil was divided into the following zones: A, soil above water-level; B, soil below water-level but with no free water surface above; C, soil below water-level with a free water surface above (Fig. 2). The category A–B was made to accommodate *A. caliginosa* and *A. terrestris* f. *longa*. In the *A. terrestris* f. *longa* and the *L. terrestris* vivaria subsidence took place leaving a gap between A and B. Worms of the species *A. terrestris* f. *longa* were frequently stretched across this air space with one end of their bodies in A and the other in B. Although there was no subsidence in the *A. caliginosa* vivarium the worms tended to congregate on the boundary between A and B, when in clay.

It will be seen from the table that, whereas *D. subrubicunda* and *L. rubellus* were entirely restricted to zone A, *A. caliginosa*, *A. chlorotica* and *A. terrestris* f. *longa* were found also in the water-saturated zones B and C, at least in certain soils. *L. terrestris* appears to avoid water strongly, for although a few specimens were found in zone C, all save two in this situation were attempting to escape up the side. In this species the diameter of the worms was only 1 mm. less than the distance between the plates, and their size relative to the apparatus was much greater than that for other species. Five specimens of *L. terrestris* meant considerable crowding, and might have resulted in the worms' occupying wetter parts than they would otherwise have done.

Table 4. *Analysis of results of choice experiments in vivaria*

Species	Soil	Distance between plates (in.)	% in A	% in B	% in C	% in A–B	% in water	% unaccounted for	Value of 100 (total worm-days)	No. of worms
A. caliginosa	Clay	¼	30	30	0	40	0	0	20	5
	Leaf-mould + clay	⅜	95·7	0	0	0	0	4·3	70	5
A. chlorotica (garden specimens), series I and II	Clay I	⅜	49·4	11·3	8·5	0	0	30	176	8
	Clay II	⅛	66·6	13·3	3·3	0	0	16·8	60	5
	Leaf-mould + clay I	¼	71·4	14·3	0	0	0	14·3	28	5→3
	Leaf-mould + clay II	⅛	97	3	0	0	0	0	34	4→3
	Sand I	¼	77	0	3	0	0	20	70	5
	Sand II	⅛	88·6	0	0	0	11·4	0	53	5→4
A. terrestris f. *longa*	Clay	⅜	37	13	12·5	30	0	7·5	120	5
D. subrubicunda	Leaf-mould	¼	86	0	0	0	0	14	100	5
L. rubellus	Leaf-mould	¼	90·4	0	0	0	0	9·6	73	4→2
	Leaf-mould + clay	¼	88·9	0	0	0	0	11·1	63	5→4
	Sand	¼	87·5	0	0	0	12·5	0	64	5→4
	Clay	¼	89·1	0	0	0	0	10·9	55	5
(acclimatized)	Leaf-mould	¼	87·5	0	0	0	12·5	0	56	5→3
L. terrestris	Clay	⅜	76·6	2·6	0	0	11·7	9	77	4

Some specimens of *A. chlorotica* and *A. terrestris* f. *longa* in zone C constructed burrows which they occupied for several days, always keeping their posterior ends out in the water above the soil, often near the aeration jet.

However, even in these species which showed the greatest tolerance of water-saturated soil, the majority of the worms were seen in the air-containing zone.

Worms on moist blotting-paper

These experiments were designed to eliminate the complicating effects of soil. The bottom of a dish was covered with blotting-paper, water was added and the dish tilted so that half the bottom was covered by water and half by moist blotting-paper. The whole was covered by an acetate sheeting box and a black cloth (Fig. 3).

The species used were *A. chlorotica*—garden and Lake Windermere specimens—and *L. terrestris*. The latter were less crowded in these experiments than in those using soil. Five worms, which were placed at water-level, were used in each experiment, and observations were made each morning and evening. The results are shown in Table 5.

The results for the Windermere specimens of *A. chlorotica* were unexpected. Approximately the same number of worm-half-days were spent out of the water, in the water, and half in the water. It had been anticipated that the worms would enter and remain in the water. Most of the garden specimens of *A. chlorotica* left the dish and escaped from the apparatus after a short time. Those remaining appeared to avoid being submerged. It may be recalled that *A. chlorotica* avoided water in sand where no humus was present (Table 4).

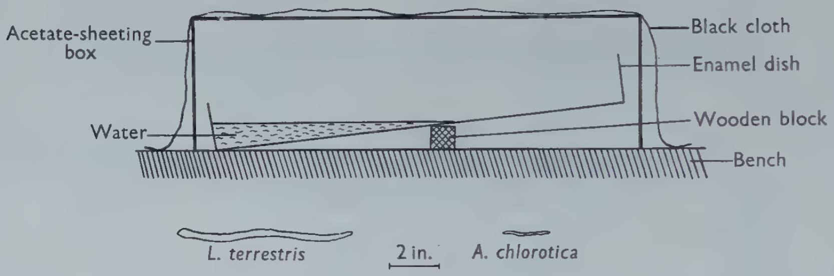

Fig. 3. Diagram to show arrangement of the covered enamel dishes. The size of the worms is shown relative to the apparatus.

Table 5. *Results of choice experiments in enamel dishes*

	In water	Out of water	Half in water
L. terrestris (tap water)	7	24	16
L. terrestris (filtered lake water)	1	24	20
A. chlorotica (from garden)	1	5	2
A. chlorotica (from Lake Windermere)	13	12	10
L. terrestris (not acclimatized)	11	17	21
L. terrestris (acclimatized)	26	6	9
A. chlorotica (from garden)	0	5	0
A. chlorotica (from garden, acclimatized)	41	0	6

Units are worm-half-days.

An almost equal number of worm-half-days were recorded for *L. terrestris* out of the water, and half in the water. There was no strong avoidance of water under these conditions, as is shown by the number of records in, and half in the water.

Wolf (1938*a*), working with *L. terrestris*, found that while most worms avoided entering tap water, they were indifferent to natural water. The possibility that London tap water also elicited an avoiding reaction was tested by making a control experiment with filtered lake water. The results were not significantly different. These experiments confirm that both species show an aversion to water in the absence of soil.

Acclimatization

Experiments were made with *L. rubellus*, *A. chlorotica* (garden) and *L. terrestris* to find out whether worms which had been kept in tap water before being put into the apparatus showed different behaviour from those which had not been acclimatized.

Five individuals of *L. rubellus* were kept in aerated tap water for 20 days before being placed in a vivarium set up with leaf-mould. All the worms, excepting two which died, behaved exactly like the unacclimatized worms (Table 4).

The experiments with *A. chlorotica* (garden) and *L. terrestris* were made using the type of apparatus of Fig. 3. Five worms of each species were kept in aerated tap water for 8 days before being placed at water level. The non-acclimatized worms were kept in pots of moist soil for a similar period. Windermere specimens of *A. chlorotica* were kept in a large tank of water in the soil. As may be seen from Table 5, acclimatization has a marked effect on these two species, the majority of the worms entering the water.

VII. DISCUSSION

A. chlorotica and *L. terrestris* are able to withstand considerable water losses, and although there is no marked difference between them, *A. chlorotica* is able to survive a slightly greater loss (75 % of the body water) than *L. terrestris* (70 %). The reverse might have been expected as *L. terrestris* is found in drier habitats than is usual for *A. chlorotica*, although it is occasionally found in very dry areas. The ability of *A. chlorotica* to survive considerable desiccation is a factor in its ability to withstand large fluctuations in the humidity of its habitat, for although the worms undergo a facultative diapause they do so in the top 6 in. of soil, not in the moist subsoil (Dr W. J. McL. Guild, personal communication), and loss of water is inevitable. The cilia of *L. terrestris* are more resistant to hypertonic media than those of *A. chlorotica* (Roots, 1956). Thus there is no correspondence between the ability of the cilia to function in hypertonic media and that of the whole worm to survive desiccation, indeed there may well be an inverse correlation.

The spreading of earthworms into new areas, by their own activity, is limited. Their maximum rate of locomotion is about 0·4 m./min., and active wandering over the surface is limited in most species (Stephenson, 1930). Earthworms are in most cases carried to new areas accidentally and small forms are more easily transferred than large ones. The distribution of a species thus depends mainly on its size and on its ability to establish itself in the new region. In the case of accidental transference to a wet or limnic locality a species which shows some tolerance of water will burrow before it can be caught by predators or killed by ultra-violet light (see below). This accounts for the frequent occurrence of *A. chlorotica* in limnic localities. It is a small worm which has been shown experimentally to have a tolerance of water and to construct burrows under water readily. Although *A. terrestris* f. *longa* shows about the same degree of tolerance of water it is only occasionally found in limnic localities. It is, however, a much larger species and therefore less likely to be carried accidentally. *A. caliginosa*, a fairly small worm, shows some tolerance of water and is sometimes found in limnic localities. Occasional records, in limnic localities, of species showing a strong avoidance of water in the behaviour experiments, e.g. *D. subrubicunda*, may be due to survival followed by acclimatization. The two terrestrial species investigated, *L. terrestris* and *L. rubellus*, both show marked avoidance of water, and although there is evidence that the former at least can be acclimatized by immersion they do not burrow when forced to live under water.

In his paper recording the occurrence of *A. chlorotica* in Lake Windermere Černosvitov (1945) remarked '...it remains to be proved by experiment whether *A. chlorotica* of Lake Windermere constitute a biological race'. No differences in the behaviour of garden and Lake Windermere specimens have been detected. Garden specimens show some tolerance of water and make burrows under water, and those from Lake Windermere do not remain exclusively in water when given a choice. Individuals from both habitats project their posterior ends from their underwater burrows. The cocoons of garden specimens of *A. chlorotica* develop under water, and produce young worms which feed and grow under water. It has already been shown that there is no physiological difference between the nephridiostome cilia of the two groups (Roots, 1956). Thus, although cocoon production under water has not been observed, it seems unnecessary to postulate that the Lake Windermere individuals constitute a 'biological race' in order to explain the occurrence of the species in that lake.

Although earthworms are able to live in water, many are found on the surface of the soil and in puddles either dead or dying after heavy rain, and this has never been satisfactorily explained. Merker (1925, 1926, 1928) and Merker & Bräunig (1927) have shown that the cause of death is ultra-violet light, but the problem of why the worms leave their burrows remains. I found that the majority of the worms investigated (including those occurring in limnic localities) avoided water when allowed a choice. Although other factors, such as oxygen lack, may contribute, this water-avoiding reaction alone would explain the worms' behaviour after heavy rain.

VIII. SUMMARY

1. The water content of *Lumbricus terrestris*, after keeping on moist filter-paper for 3 or 4 days, is 84·8 % of its body weight. That of *Allolobophora chlorotica* is 80 % of its body weight. Both species can survive a loss of 60 % of the body weight, but not much more.

2. Earthworms of the species *A. chlorotica*, *A. terrestris* f. *longa*, *Dendrobaena subrubicunda*, *L. rubellus* and *L. terrestris* are all able to survive from 31 to 50 weeks in soil totally submerged beneath aerated water. The same species, and *A. caliginosa* can survive for 72–137 days in aerated tap water without food.

3. Garden specimens of *A. chlorotica* make U-shaped burrows in soil beneath water. They do not irrigate either the burrows or glass tubes. Egg-cocoons of *A. chlorotica*, taken from culture pots of soil, will hatch under water and the young worms will feed and grow though totally immersed.

4. *D. subrubicunda, L. rubellus and L. terrestris* avoid immersion in water when allowed a choice between water-saturated and moist but air-filled soil. When *A. caliginosa*, *A. chlorotica* and *A. terrestris* f. *longa* are allowed a similar choice, a small proportion of the population is always found in the water-filled soil.

5. Enforced submergence before being allowed a choice of environment does not affect the behaviour of *L. rubellus*, i.e. water is still avoided. Similar treatment of *A. chlorotica* and *L. terrestris* results in a more frequent choice of water.

6. It is unnecessary to postulate that the individuals of *A. chlorotica* occurring in Lake Windermere constitute a 'biological race'.

7. The avoiding reaction of earthworms to immersion may explain why they leave the soil after heavy rain.

I wish to thank Prof. G. P. Wells, F.R.S., for his advice, criticism, encouragement and supervision of the investigation; the Committee of University College, London, for the Margaret Browne Studentship which made the work possible; and the Trustees of the British Museum (Natural History) for placing the collections and manuscripts of the Museum at my disposal.

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THE QUANTITATIVE NUTRITIONAL REQUIREMENTS OF *DROSOPHILA MELANOGASTER*

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I. INTRODUCTION

Drosophila melanogaster was the first multicellular animal to be reared under aseptic conditions and on a diet of known composition (see Trager, 1947). The qualitatively adequate food then used has been improved from time to time (Schultz, St Lawrence & Newmeyer, 1946; Begg & Robertson, 1950). Details of the most recent modifications are summarized by Hinton, Noyes & Ellis (1951) who, nevertheless, conclude that 'no claim can be made that all the ingredients are necessary or in the proper concentration in the medium as now constituted'. The purpose of this paper is to define a diet which contains only necessary ingredients in amounts which give the best possible growth and development. To do this, dose-response curves have been obtained for all the major components of the food under conditions in which any interactions between them are likely to be of minor significance.

It is impossible, of course, to devise a food medium which will permit normal growth of all strains of *D. melanogaster*, since different strains are known to have different nutritional needs (Schultz & Service, 1951; Sang, 1954) and also different synthetic abilities (Hinton, Ellis & Noyes, 1951). However, synthetic culture media in current use are very inadequate when measured by the time taken for larval development: for instance, Hinton (1955) reports a larval period of 10·8–11·5 days on his medium whereas the duration of this stage is between 4·0 and 4·5 days when live yeast is supplied as the food. Such a sub-optimal diet may seriously disturb the metabolic pattern of the developing larvae and an improved medium which minimizes this is likely to make work, say on the effects of chemicals (Herskowitz, 1951), more exact than it can now be. Further, a detailed examination of the requirements of one strain should show how close it is possible to get to an ideal diet, give a measure of the changes needed to correct this diet for other strains, and, perhaps, also indicate the constituents most likely to require such a quantitative adjustment.

Differences of nutritional requirements imply adaptation to this primary component of the environment, and da Cunha, Dobzhansky & Sokoloff (1951) have shown that the relative success of this or that strain of *D. pseudo-obscura* depends partly on the quality of the food (yeasts and bacteria) available to them when they are in competition with each other. This clear demonstration of the effect of nutritional variables on ability of different genotypes to survive under conditions of intraspecific competition also suggests that the precise determination of the minimal nutritional needs of a species is of some importance for our understanding of micro-evolution. This point has generally been ignored due to the well-recognized ability of animals to survive under patently adverse nutritional conditions; yet it is a commonplace of all plant and animal husbandry that related species, and many strains within species, have very precise minimal food requirements for normal growth. It is, therefore, also of some interest to show what these minimal needs are for an insect so commonly used in experimental biology.

II. MATERIALS AND METHODS

The two main technical problems which had to be solved at the start of this work were: the sterilization of large numbers of eggs at one time and the formulation of a diet which was balanced in the sense that no one constituent was present in such sub-optimal amounts that it was likely to influence requirements for other components. The latter point was met by testing a series of experimental diets each approximating more closely to the final balanced formula, since this seemed the method least open to objection (see § III). The first problem involved developing the method of egg sterilization which follows.

(*a*) *Sterilization methods*

Numerous techniques have been described for sterilizing insect eggs (Glaser, 1923; Hinton, 1955) but nearly all of these prove ineffective when large numbers are handled. Begg & Sang's (1950) method was used in a preliminary series of experiments not reported on in detail here, but occasionally it failed completely. At the time the reasons for these failures were not at all clear and the problem of devising a satisfactory sterilization technique had to be re-examined.

The normal *Drosophila* culture contains moulds and bacteria as well as the yeasts with which it is seeded, and all of these are usually found adhering to the sculptured exochorion of the eggs. This symbiotic relationship between the fly and the micro-organisms on which it feeds has generally been overlooked and it is perhaps worth recording that 3-day-old flies will still lay eggs with yeast cells adhering to them 2 days after these flies have been removed from any contact with yeasts. The problem of egg sterilization thus resolves itself into killing or otherwise removing the adhering micro-organisms, or of breaking this symbiotic relationship. While it is possible to rear adult flies under aseptic conditions it is difficult to collect eggs from them. The fertility of inbred strains, normally poor in any event, is markedly lowered under such conditions and the regular collection of large numbers of eggs becomes too cumbersome a procedure to be practical. Eggs have to be collected from adults reared on the usual maizemeal-molasses medium seeded with live yeast. Many antiseptics were found to be effective against the bacteria and growing yeast cells carried over from the medium; none of them would kill the spores, at least not in concentrations which were not equally toxic to the eggs. This resistance of the spores, and the fluctuations of the proportions of spores found in cultures, appears to explain the variations of success encountered when using Begg & Sang's (1950) sterilization method noted above.

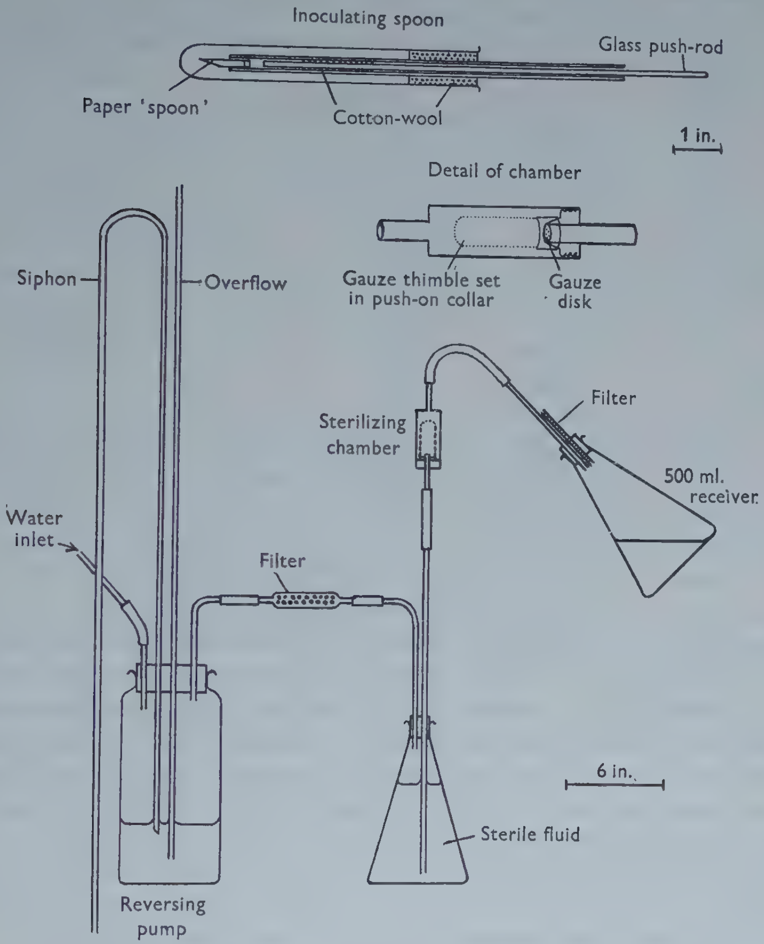

Fig. 1. The sterilizing apparatus consists of two parts, a reversing water pump on the left which is connected by way of a sterilized cotton-wool filter to the flasks, and a sterilizing chamber on the right. The sterilizing fluids are pushed over the eggs contained in the chamber by the air pressure developed in the pump, 20–30 ml. of the fluid being driven into the sterile receiving flask at a time. When the siphon breaks, the fluid in the tubes is sucked back and the eggs again redispersed within the stainless steel gauze thimble. Details of the metal chamber and of the gauze thimble are given in the upper part of the figure on a scale approximately three times that of the rest of the diagram.

The third possible method of sterilizing eggs gives satisfactory results. It depends on chemical dechorionation of the eggs followed by a washing procedure sufficient to ensure that any partially dissolved chorions are removed. Any bacteria not killed during the dechorionation are usually washed clear of the eggs or killed by Cetavlon, which is an antiseptic as well as a detergent. Occasionally parent cultures become contaminated with *Bacillus subtilis* and spores of the micro-organism may be so lodged as to resist the treatment; but this source of infection can generally be shaken off by transferring the flies to fresh culture bottles. The details of the sterilization routine are:

(1) Collect eggs on 2% agar in watch-glasses (see Begg & Sang, 1950) and brush off into a beaker.

(2) Dechorionate eggs by covering with fresh, filtered 3% solution of chloride of lime.

(3) Decant the surplus hypochlorite solution after 20 min. and wash the eggs with water.

(4) Transfer eggs to the wire thimble of the washing apparatus (Fig. 1) and wash with sterile water, 2% Cetavlon (Cetrimide, I.C.I.), and again with sterile water.

(5) Transfer eggs under aseptic conditions, using sterile paper spoons, to sterile agar plates.

The washing procedure takes about 1 hr. and the entire sterilization about 2 hr.

The eggs used in the experiments described here were collected during a 4 hr. period from pure line Oregon S flies which had been brother-sister mated for over eighty generations before the work started. Once sterilized and transferred to the sterile agar plates they were kept at 25° C. for 20–22 hr., and the experimental cultures were set up using the larvae which had hatched by that time. Initially, these newly hatched larvae were transferred to the media under test using sterile platinum spoons, but variability in survival, particularly due to handling differences between operators, showed that this method was inadequate. A sterile paper spoon of the kind shown in Fig. 1 was therefore devised and this was employed in all the work reported. Using this method, about 2000 larvae could be set up in an hour. A separate experiment showed that the optimum number of larvae per culture was about forty under our conditions (Fig. 2), and this was adopted as standard. Each treatment was tested in duplicate, or in triplicate where the mortality was expected to be high. As an extra precaution, cultures were set up under the cover of a small chamber which was sterilized by an ultra-violet lamp. The rate of infection found for the first thousand cultures set up using this method was 4·7%.

(*b*) *Culture medium*

A preliminary series of experiments was run to determine the approximate optimal amounts of the various dietary constituents, and this gave a standard medium 'A' which is detailed in Table 1. It was hoped that this medium would be sufficiently near the optimum to eliminate any gross interactions between components. Five ml. of the medium was autoclaved for 15 min. at 20 lb. pressure, and then cooled with shaking to disperse the casein evenly in the 1 in. boiling tubes used. The cultures were not sloped. Innoculated cultures were then kept at 25° C. and at 80% relative humidity.

Table 1. *Composition of standard test media*

	Medium A (g.)	Medium B (g.)	Medium C (g.)
Agar (Oxoid, Kobe no. 1)	3·00	3·00	3·00
Casein (Genatosan, low vitamin)	4·00	5·00	5·50
Fructose	1·00	0·75	0·75
Cholesterol	0·02	0·015	0·03
Lecithin	nil	0·20	0·40
Yeast nucleic acid	0·25	0·40	0·40
Aneurin (Thiamine)	0·0002	0·0001	0·0002
Riboflavine	0·0003	0·0002	0·0010
Nicotinic acid	0·0005	0·0001	0·0012
Ca pantothenate	0·0004	0·0003	0·0016
Pyridoxine	0·0001	0·00004	0·00025
Biotin	0·00004	0·000006	0·000016
Folic acid	0·0001	0·00006	0·0003
Choline	0·006	0·004	nil
Thymine	0·0002	nil	nil
$NaHCO_3$	0·126	0·126	0·140
$FeSO_4, 7H_2O$	0·002	0·002	nil
$CaCl_2$	0·002	0·002	nil
$MgSO_4$	0·002	0·002	nil
$MnSO_4, 4H_2O$	0·020	0·020	nil
NaCl	0·002	0·002	nil
KH_2PO_4	0·183	0·183	0·183
Na_2HPO_4	0·189	0·189	0·189
Water to	100 ml.	100 ml.	100 ml.

(*c*) *Measurement of performance*

Rate of growth has generally been adopted as the most suitable measure of the adequacy of a diet, and in work with insects growth rate has usually been measured by the time spent in egg, larval and pupal stages, taken together. For most purposes this measurement of the duration of pre-adult life gives sufficient information concerning the adequacy of a diet, but for a holometabolous insect it is desirable to relate nutritional effects only to the duration of the larval stage since growth is restricted to these instars. Since only newly emerged larvae were set up in the experiments described, the duration of the egg stage is automatically excluded from the data, and initially development was measured by timing the formation of pupae. Unfortunately, it is difficult to do this using synthetic media, whereas it is easy to count emerged adults. Counting adults has also the advantage that cultures remain unopened for 10–12 days, which gives any slowly developing infections time to become obvious and so limits the number of subculture tests needed to check for such contaminations. However, the distribution of adult emergence times is not normal (Maynard Smith & Maynard Smith, 1954) and is complicated by a difference in the development time of the two sexes (Bonnier, 1926) and a marked diurnal rhythm in the time of eclosion (see Pittendrigh, 1954, for references). These complications cannot be overcome completely, but their importance is reduced somewhat if the duration of the pupal stage (4·3 days) is subtracted from the eclosion time data, and the logarithm of the synthetic 'larval period' so obtained is used as the statistic measuring development time. These values have been used throughout this paper. Under sub-optimal nutritional conditions size may be reduced and survival lowered, and account is taken of such changes whenever they add to the description of responses to the different nutrients examined.

Contrary to expectation (Sang, 1949), Fig. 2 shows that there is an optimal number of larvae which should be seeded on to the synthetic medium (between forty and fifty per culture) and that survival as well as development rate is modified when other levels of crowding are used. So forty larvae per culture was adopted as the standard innoculation and cultures were discarded if they showed that a smaller number than this had actually been placed on the medium. No data from cultures showing any sign of contamination by bacteria or moulds are included in the tables and figures presented.

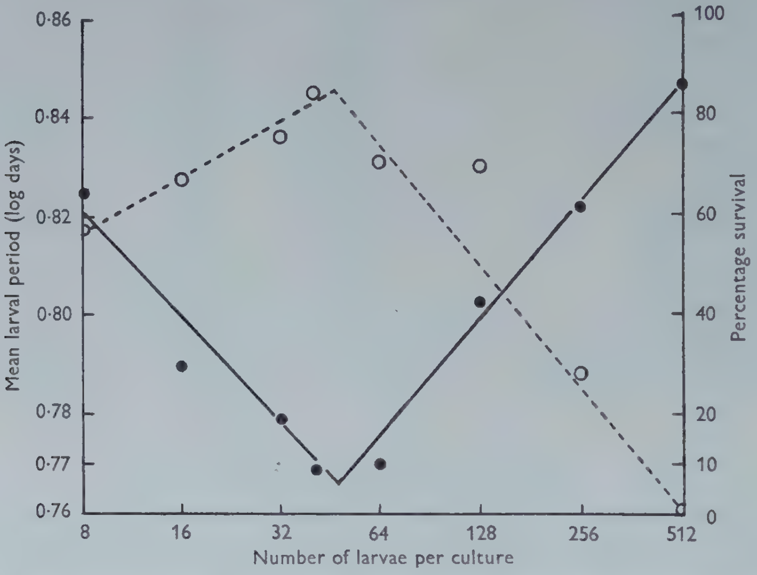

Fig. 2. The effect of crowding on rate of development (lower graph) and on survival (upper graph). Although growth was timed by counting the emergence of adults twice daily, these times have been adjusted by subtracting the 4·3 days of the pupal period to give the computed larval periods used above. A logarithmic plot of the data then shows a nearly normal distribution of the calculated pupation times, so the logarithm of the duration of the larval periods is used here, and in subsequent figures, as the statistic measuring growth rate.

(d) *Uncontrolled variables*

As the work proceeded, it was found that the standardization of procedures just outlined did not eliminate all the variability between experiments, and separate tests showed that some of the differences derived from the condition of the parent flies from which the eggs were collected (Table 2). Within the range tested (Table 2A) there was no evidence that the conditions under which successive batches of adults had themselves developed made any significant difference to the rate of growth of their progeny ($F = 2{\cdot}58$, D.F. 1 and 672, $P > 0{\cdot}5$). Nor was there evidence that even considerable delays in setting up the larvae after they had hatched (Table 2 D) influenced the time they took to develop, provided this was measured from the hour of hatching. On the other hand, ageing of the parents had a significant influence on the development of their progeny (Table 2 B), larvae developing from eggs laid by flies in the middle of the age range tested taking longest to develop. Deviations from the best linear regression line give $F = 9{\cdot}6$, D.F. 6 and 1311, and $P < 0{\cdot}01$. Similarly, the nutrition of the parent flies when adult (Table 2 C) affects the development of the larvae they produce: live baker's yeast proving to be a better adult food than the same yeast killed by autoclaving or than brewer's yeast. ($\chi^2 = 10{\cdot}29$, D.F. 4 and $P < 0{\cdot}05 > 0{\cdot}02$ when baker's and brewer's yeasts are compared.)

Table 2. *Uncontrolled variables affecting larval development*

	Mean larval development time (log days)	*s*	*n*
A. Day on which parents hatched from cultures:			
1st day emergents	0·779	0·053	116
2nd day emergents	0·740	0·045	137
4th day emergents	0·778	0·045	133
6th day emergents	0·760	0·055	141
8th day emergents	0·753	0·052	89
B. Age of adults (days):			
2	0·668	0·050	172
3	0·666	0·046	130
4	0·671	0·050	164
5	0·694	0·049	181
6	0·675	0·032	167
8	0·686	0·031	174
9	0·671	0·046	150
11	0·661	0·048	181
C. Adult nutrition:			
Live baker's yeast	0·741	0·053	168
Live brewer's yeast	0·755	0·068	150
Heat-killed baker's yeast	0·753	0·058	166
D. Age of larvae when placed on the medium (hr.):			
0–0·5	0·732	0·052	130
2·5–3·0	0·725	0·046	90
5·0–6·0	0·727	0·047	123
8·0–9·0	0·724	0·055	95

The four tests were carried out at different times using different parent flies. Media for A and B were refrigerated for 1 week prior to the tests in order to minimize any medium ageing effects. Adults used in A, C and D were kept for 3 or 4 days before eggs were collected from them. *s* is the standard deviation in log days for the *n* individuals which hatched from the cultures.

Some of these variables could be controlled by ensuring that adults were fed in a standard fashion and larvae set up within an hour or two of hatching, but it proved impractical to restrict egg collections to flies of a particular age. Further, as comparison of A and B of Table 2 shows, some generations of flies produced larvae which grew faster than others, presumably because the parents had been exceptionally well nourished as larvae or for some other reason which could not be controlled. (See Durrant (1955) for a possible cytoplasmic cause of this variability.) Standard cultures were therefore also run in parallel with experimental cultures whenever it was necessary to check the effect of these uncontrolled variables.

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III. EXPERIMENTAL

Although something is known of the interactions between dietary constituents in the nutrition of higher animals, it could not be assumed that they would necessarily hold for a holometabolous insect which devotes one stage of its life-cycle almost exclusively to feeding and growth. Medium A (Table 1) was devised, therefore, as a nearly optimal diet in the sense that each constituent was present only slightly in excess of an experimentally determined minimum. The preliminary series of experiments which led to the formulation of medium A was necessarily only approximate, and it is not detailed here. The experiments done using medium A were designed to give more precise information concerning these minimal needs and it was possible to construct a more satisfactory minimal formula from the results (medium B, Table 1). Whenever necessary this was refined still further as a third step (medium C, Table 1). The results show that each successive improvement led to more rapid and normal growth and with this came a decrease of variability and greater precision in the dose response curves. Data from the two main series of tests, based on medium A and medium B respectively, will be given together in order to simplify the presentation.

(*a*) *Protein requirements*

Casein was one of the first substances added as a supplement to the early killed yeast *Drosophila* diet (Northrop, 1917; Bacot & Harden, 1922). Later workers tended to use casein hydrolysate as the sole protein source, either supplemented with tryptophane (Tatum, 1939) or with tryptophane and cystein (Lafon, 1938) to make up for the partial destruction of these amino acids during the hydrolysis process. First attempts to substitute pure amino acids for this hydrolysate were unsuccessful (Lafon, 1938; Chu, 1945) but Schultz *et al.* (1946) accomplished the substitution using only L-amino acids. Even so, addition of whole casein to their mixture improved growth, suggesting that some polypeptide present in casein had a beneficial effect on development. Hinton, Noyes & Ellis (1951) confirmed that a mixture of L-amino acids could replace casein. Three questions are raised by this work: do current diets supply the optimal amount of protein? do the proportions of amino acids present in casein represent a balanced supply of each? and does casein still appear to supply some essential polypeptide when the optimal amount of each L-amino acid is provided?

The last question can be given only a partial answer here since available L-amino acids were found to be impure and contaminated with heavy metals. Even apparently pure amino acids gave poor growth (see below) and their use had to be abandoned on that account. For our purposes casein hydrolysate had no advantages over purified casein, so the experiments were done using the latter. The dose-response curves for casein are shown in Fig. 3.

Following Tatum (1939), most workers have used 2 % casein in the diet (Begg & Robertson, 1950), or about the same amount of hydrolysate (Villee & Bissell, 1948), or of its equivalent in amino acids (Hinton, Noyes & Ellis, 1951). Our results show that this is insufficient for good development and that the optimum amount of casein in the food is around 5%. Development is retarded if less protein than this is supplied; excessive amounts of protein also slow growth though only to a small extent within the range tested. At 1% casein mortality is all during the larval stage; 2% casein is sufficient to overcome this loss and survival is constant at this and higher concentrations. As judged by the average weight of males, all flies hatching from the series of medium B cultures are the same, except for those reared on the highest amount of protein which are about 10% lighter than average. On a low protein diet, then, development is prolonged until sufficient protein is accumulated to allow the larvae to reach normal size, whereas excess protein upsets this normal metabolic balance.

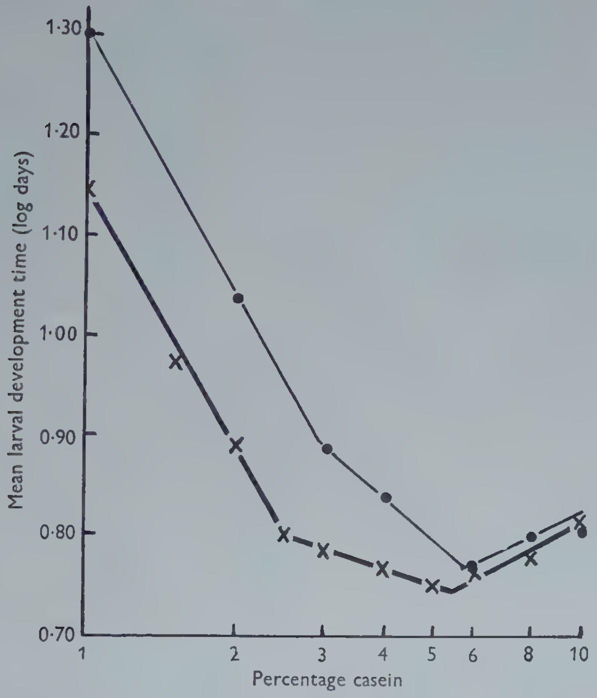

Fig. 3. Responses to various amounts of casein in the diet: upper curve when medium A is used and lower curve using Medium B. The supply of casein is set out on a logarithmic scale in order to demonstrate the points of inflexion of the curves more precisely.

It does not follow from this that casein is qualitatively complete for *Drosophila* and comparison of the amino acid compositions of casein and yeast (Block & Bolling, 1947; Lindan & Work, 1951) suggests that casein may be deficient in any or all of the following indispensable or partly indispensable amino acids: arginine, cystine, glycine, histidine, lysine, threonine and tryptophane. A preliminary test showed that only arginine, cystine and tryptophane were likely to improve growth, and a further test was run using these singly and in combination (Table 3). This showed that addition of these amino acids to casein did not improve the diet in any way.

As already noted, an attempt to replace the casein with commercially available L-amino acids resulted in a medium which was toxic to the larvae. However, by courtesy of Dr T. Hinton and Difco Laboratories, it was possible to try out their experimental medium K 115225 which had the same composition as the food used by Hinton, Noyes & Ellis (1951). This medium contained just over 2 % amino acids, so it was also tested with all constituents at twice the normal concentration in order to bring the amount of amino acids nearer to our optimum. This concentrated medium showed some improvement over the original (Table 4) which could not all be attributed to its firmer consistency since addition of agar alone resulted in only a slight decrease of development time although it led to a notable improvement of survival. On the other hand, addition of casein to the Difco medium produced a marked improvement of growth rate (Table 4). This, at least, does not contradict Schultz *et al.*'s (1946) conclusion that casein supplies some polypeptides which are used as such by *Drosophila*.

Table 3. *Addition of amino acids to the complete casein medium*

Medium composition	Mean larval development time (log days)	*s*	*n*
Control, medium B	0·717	0·054	95
plus 1 % L-arginine	0·714	0·042	97
plus 2 % L-tryptophane	0·718	0·079	89
plus 1 % L-cystine	0·770	0·058	83
plus arginine and tryptophane	0·742	0·064	64
plus arginine and cystine	0·734	0·041	93
plus tryptophane and cystine	0·707	0·069	58
plus arginine, cystine and tryptophane	0·713	0·068	64
Control, pasteurized	0·682	0·036	160

Table 4. *Comparison of amino-acid medium with standard casein medium*

	Mean larval period (log days)	*s*	*n*	% survival
1. Control, medium B	0·719	0·049	158	79
2. Difco amino-acid medium	0·948	0·029	93	46
3. Concentrated Difco medium	0·905	0·042	51	64
4. Difco medium with double agar	0·947	0·037	120	60
5. Difco medium with 2·5 % casein	0·836	0·037	81	67

(*b*) *Carbohydrate requirements*

Hassett (1948) has reviewed the work done on the carbohydrate requirements of insects, and measured the food value of an extensive range of sugars and related substances in the nutrition of the adult *Drosophila*. When fed in equivalent solutions, the usefulness of the common sugars to adult flies is: fructose > maltose > sucrose > glucose > galactose > xylose > lactose. He has also shown that larvae develop faster on a sterile yeast 'starvation' diet when this is supplemented with fructose rather than with sucrose or glucose. This indication that *Drosophila* is adapted to fructose and not to the common physiological sugar, glucose is probably not surprising if we remember that both stages normally feed on fruit or plant exudates (Gordon, 1942; Wagner, 1944) infected with wild yeasts. However, there is another possible explanation of the results of these experiments on larval nutrition. When proteins and sugars are autoclaved together some of the amino acids are inactivated (Evans & Butts, 1949) and the nutritional value of the whole is lowered. This effect is clearly shown in Table 3; pasteurized media give faster growth than autoclaved media. The various sugars react differently in this respect and show this grading: fructose < maltose < lactose < glucose < xylose (Lewis & Lea, 1950). As this sequence corresponds almost exactly with Hasset's (1948) it was necessary to check that his results were not due to such a protein-sugar reaction; and this was done by adding a sterile solution of the sugar to the medium after autoclaving (Table 5). The results show that fructose is the sugar most suited to *Drosophila* and all experiments were done using it. Except for maltose, which is apparently not used by the larvae, the nutritional value of these sugars corresponds to that found by Hassett (1948) for adults.

Table 5. *Influence of feeding different sugars on larval development*

	Mean larval period (log days)	s	n
Control—autoclaved with fructose	0·736	0·049	92
Without sugar	0·794	0·069	74
Fructose added after autoclaving	0·732	0·036	93
Maltose added after autoclaving	0·803	0·053	21
Lactose added after autoclaving	0·767	0·057	90
Glucose added after autoclaving	0·747	0·046	53
Starch added after autoclaving	0·760	0·062	64
Sucrose added after autoclaving	0·768	0·044	64

Dose-response curves were determined for fructose, sucrose and lactose, and all showed optimal development at about 0·8 % sugar. Only data for this concentration are given above.

Apparently no work has been done to find the optimal amount of sugar in the *Drosophila* diet, so more detailed dose-response curves were determined for the two standard media (Fig. 4). On both media, the optimal amount of fructose was found to be near 0·75 g.%, as judged by rate of development. Absence of sugar from the diet slows development but only to a small extent under the better conditions of medium B; excess of sugar also has a deleterious effect on growth rate. Neither of these circumstances affects survival. On medium A, maximum size is reached when about 2 % fructose is supplied (Fig. 4) but on medium B only larvae reared without sugar are smaller than normal. As Rudkin & Schultz (1949) have also noted, response to sugar therefore depends partly on the composition of the rest of the medium.

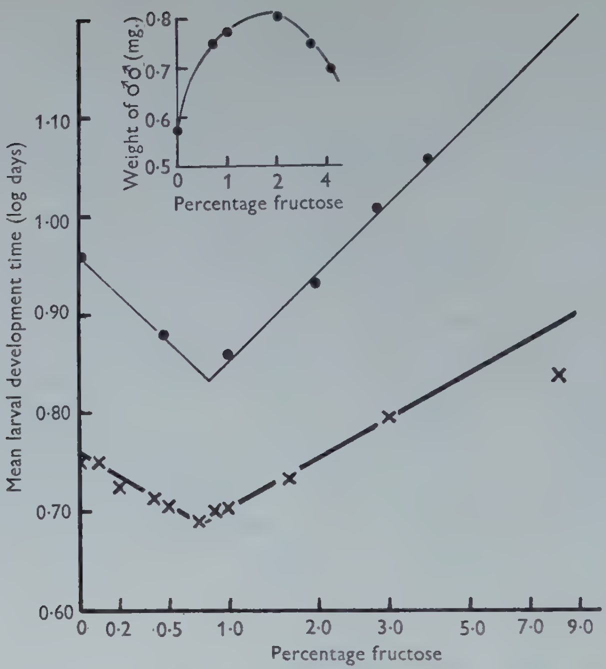

Fig. 4. Responses to different concentrations of fructose in the diet: upper curve medium A, lower curve using medium B. A logarithmic scale is again used, and the zero concentration has been included, although it is not to scale. The small inset figure shows the average size of males emerging from the medium A cultures.

(*c*) *Fat requirements*

Insects differ from vertebrates in not requiring fat soluble vitamins and they seem to need only one fat-like substance, cholesterol, in their diet (Trager, 1953). This has been met in the *Drosophila* medium by incorporating from 0·01 % (Villee & Bissell, 1948) to 0·1 % (Begg & Robertson, 1950) of cholesterol, or cholesterol and ergosterol. There is no evidence that ergosterol is in any way superior to cholesterol, or that it performs any function not met equally well by cholesterol, and for this reason we have determined the requirements only of the latter substance (Fig. 5).

Larvae cannot grow without an external supply of cholesterol. The optimum amount of cholesterol is not sharply defined and lies between 0·01 and 0·05 %. Survival and size are but little affected within the range covered by Fig. 5 and only the lowest amount (0·001 %) causes more than a 10 % increase in larval mortality. Excess cholesterol affects only rate of development.

Lafon (1937) reported that lecithin was also essential for *Drosophila*, but as his medium contained no cholesterol, it seems probable that this latter, and essential, substance was present as a contaminant of his 'egg lecithin' giving a misleading result. Such a contamination is certainly found in the samples of commercially available egg lecithin we have examined. As Schultz & Rudkin (1949); Begg & Robertson (1950); Hinton (1952) do not include lecithin in their media, it is clear that this substance is not essential for *Drosophila* and our findings confirm this. Addition of purified lecithin does speed up larval development, however, and there is a distinct optimal supply under our conditions (Fig. 6). For this reason lecithin was incorporated in medium B. Even a great excess of lecithin cannot substitute for cholesterol, as one would expect, but we have been unable to show if it has any sparing action on cholesterol requirements.

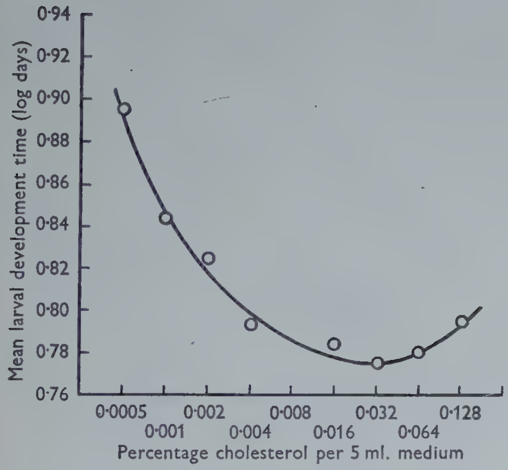

Fig. 5

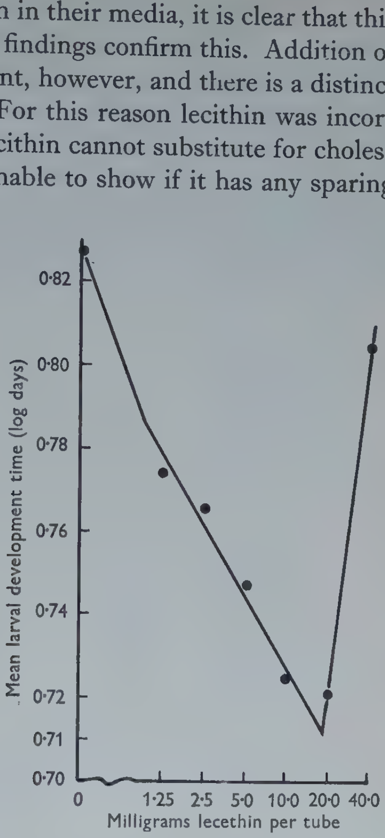

Fig. 6

Fig. 5. Responses to different amounts of cholesterol. Cholesterol is insoluble in both media and great difficulty was experienced in ensuring that it was properly dispersed after the cultures were autoclaved. The presence of lecithin in medium B increased this difficulty since these two substances tended to aggregate together at the sides of the culture tubes. Lecithin was omitted from cultures described above for this reason.

Fig. 6. Addition of Lecithin to medium A. Note that the zero point is not to scale and that medium A already contains choline in an adequate amount.

Since lecithin is a source of choline, it is worth noting here that the response curve shown in Fig. 6 was obtained using a medium which already contained a little more than an optimal supply of choline (Fig. 13). It follows that the lecithin must supplement some synthetic processes other than those involving choline.

(*d*) *Nucleic acid requirements*

Following Schultz *et al.*'s (1946) demonstration that Ribose Nucleic Acid acted as a growth factor for *Drosophila*, Villee & Bissell (1948) showed that the growth promoting effect lay 'not in the RNA as such, but in the purines and pyrimidines, especially adenine', as one might expect. That is, wild type strains have a limited ability to synthesize their own nucleic acids, but they can grow normally only if an external supply of purines and pyrimidines is available to them. Some mutant strains have lost this synthetic power (Hinton, Ellis & Noyes, 1951; Hinton & Roberts, 1952) and cannot grow without an external supply of one or other of the purines or pyrimidines found in RNA. Desoxyribose nucleic acid, on the other hand, inhibits larval growth (Schultz *et al.* 1946) although thymine itself appears to have some growth promoting abilities (Begg & Robertson, 1950). Here we shall be concerned with only two problems relating to nucleic acid supplies: what the optimum amount of RNA is for the diet, and whether or not RNA in optimal supply needs to be supplemented by the addition of any particular purine or pyrimidine.

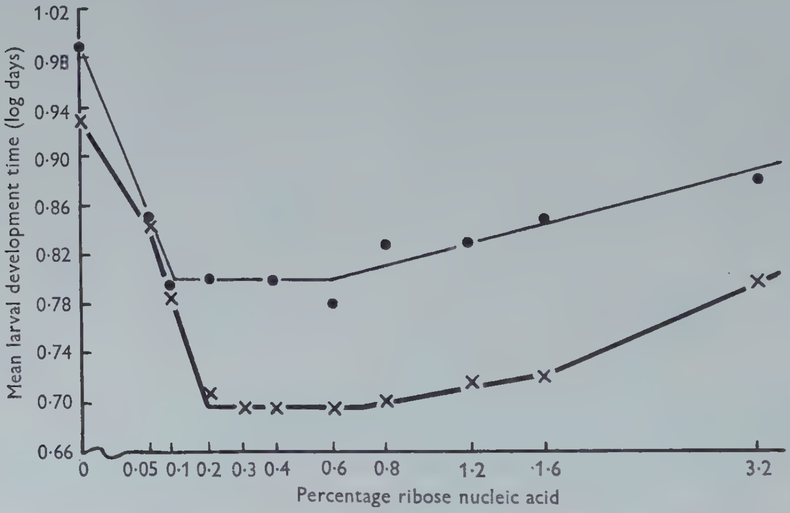

Fig. 7. Response to R.N.A. when added to medium A (upper curve) and to medium B (lower curve). The R.N.A. used was brought into solution with sodium bicarbonate before it was added to the medium. As before, the zero point is not to scale.

Fig. 7 shows that excess ribose nucleic acid in the diet is detrimental to growth and that the optimum supply under our conditions lies between 0·3 and 0·6 %. This should be compared with the 0·1 % used by Villee & Bissell (1948). Survival is little affected by omission of nucleic acid from the food, but development is then prolonged by about 3·5 days when medium B is used. If 0·4 % ribose nucleic acid is included in this diet, addition of any one of the purine or pyrimidine bases results in no further improvement of growth rate or of survival (Table 6). Indeed, both adenine and thymine slow growth and lower survival, and supplementary thymine should be omitted from the diet for this reason. An optimal supply of ribose nucleic acid is all that is necessary in the food.

(*e*) *Vitamin requirements*

Like other insects, *Drosophila* needs only vitamins of the B group (Trager, 1953). These are usually all included in the synthetic food, but in amounts which differ from formula to formula. As Begg & Robertson (1950) have emphasized, there is no clear evidence that all of them are essential to the diet or that they are present in optimal amounts. The first point is a particularly pertinent one since Leclercq (1948) has shown that *Tenebrio* requires only six of the dozen or so B vitamins. We have thus to find if any vitamins can be dispensed with, and what the optimum supply is for the remainder.

Table 6. *Addition of Purine and Pyrimidine bases to medium containing optimal R.N.A.*

	Mean larval period (log days)	*s*	*n*	% survival
1. Control, medium B	0·695	0·042	87	72·5
2. Adenine added at 0·1 %	—	—	0	nil
3. Guanine added at 0·1 %	0·695	0·042	78	65·0
4. Cytosine added at 0·1 %	0·681	0·045	99	82·4
5. Uracil added at 0·1 %	0·799	0·049	57	84·0
6. Thymine added at 0·1 %	1·049	0·049	13	11·0

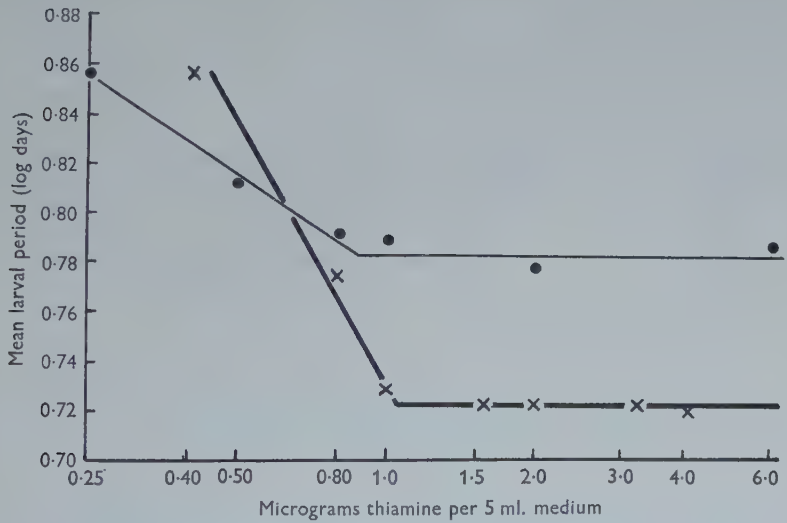

Fig. 8. Response to thiamine in medium A (upper curve) and medium B (lower curve). There was no survival of larvae reared on media containing no thiamine.

I. *Thiamine* (*Aneurin*). Using an autoclaved yeast medium, Van't Hoog (1935) showed that *Drosophila* larvae could not grow and pupate without addition of vitamin B_1 to this food. Lafon (1937) and Schader (1941), on the other hand, did not find this vitamin to be essential, and the latter worker claimed that its omission merely delayed pupation and emergence by a day. This contradiction was resolved by Hinton, Noyes & Ellis (1951) using highly purified materials; they confirmed Van't Hoog's (1935) results, and our data (Fig. 8) are in agreement with this. Thiamine is an essential constituent of the *Drosophila* diet and a little over 1 μg. per 5 ml. of medium is necessary for normal development. Less than this amount slows larval growth, but as little as 0·4 μg. per 5 ml. medium is sufficient to allow larvae to grow, pupate and emerge without any increase of total mortality. Larvae fed at this level become normal-sized flies. Excess of this vitamin has no effect on development, size or survival, within the range tested, so that media containing from 1·2 to 12·0 μg per 5 ml. will be satisfactory.

II. *Riboflavine.* Van't Hoog (1935) also showed that riboflavine was essential for the development of *Drosophila* but this was not confirmed by Lafon (1937) or by Tatum (1939), although addition of it to their media did improve the growth rate. Hinton, Noyes & Ellis (1951) were again able to show that Van't Hoog's conclusion was correct, and this is also in accord with our results (Fig. 9). No pupae are formed unless riboflavine is provided in the diet and at least 4 μg. per 5 ml. of medium must be supplied to give normal growth and survival. Below about 2 μg per culture, size is reduced and survival of both larvae and pupae is lowered. The amount of riboflavine in an optimal diet should therefore exceed 4 μg. per 5 ml. of medium.

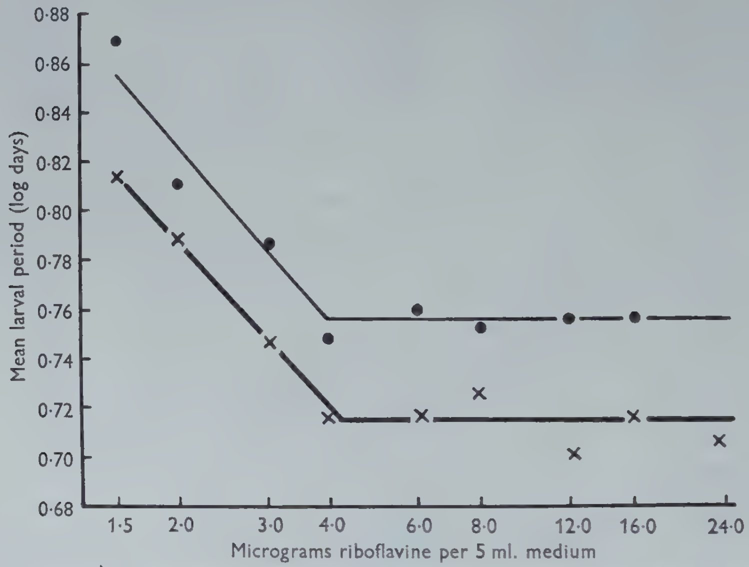

Fig. 9. Response to riboflavine when added to medium A (upper curve) and to medium B (lower curve). As in preceding figures, the dose scale is logarithmic.

III. *Nicotinic acid.* Nicotinic acid has been found to be essential for pupation (Hinton, Noyes & Ellis, 1951) and its addition to an incomplete medium accelerates larval development (Tatum, 1939). Our data show (Fig. 10) that larvae die if deprived of nicotinic acid and that a small amount (1 μg. per 5 ml. medium) is just sufficient to allow some undersize and inviable pupae to form. Nicotinic acid deficiency has effects similar to those found with riboflavine. The minimal supply for normal growth and survival is about 6 μg. per 5 ml. of medium and at least as much as four times this amount can be fed with some small improvement in growth rate.

IV. *Pantothenic acid.* Schultz *et al.* (1946) were the first to include pantothenic acid in the medium, but they give no data concerning its effects. Hinton, Noyes & Ellis (1951) showed that larvae could live for up to 29 days without this vitamin, but they then grew only slowly and failed to pupate. Our experiments confirm these findings, and further demonstrate that about 3 μg. per 5 ml. medium is necessary for pupation (Fig. 11). Nearly 10 μg. per culture is required to give optimal larval growth: twice this amount leads to no further improvement.

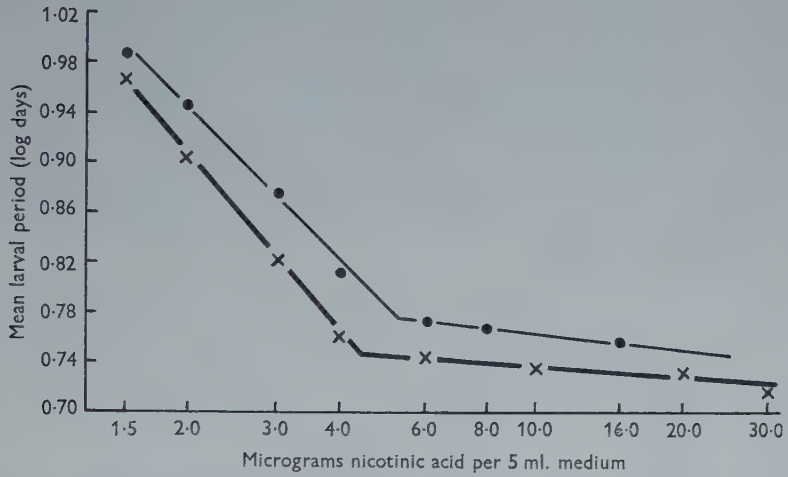

Fig. 10. Response to different quantities of nicotinic acid in medium A (upper curve) and medium B (lower curve). There is no survival if this vitamin is omitted from the diet and only a few stunted pupae form if only 1 μg is supplied per culture.

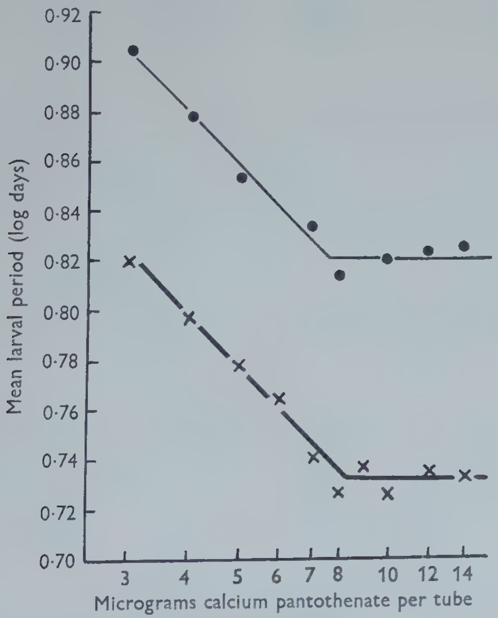

Fig. 11. Response to various amounts of calcium pantothenate in medium A (upper curve) and medium B (lower curve). No adults were formed when less than 3 μg. Ca pantothenate was incorporated in the medium.

V. *Biotin*. Hinton, Noyes & Ellis (1951) have shown that biotin is essential for larval development and for pupation. About 0·08 μg. per 5 ml. medium proved to be the minimal supply under their conditions and as much as 0·5 μg. per 5 ml. medium had no toxic effect. Our dose response curves give practically the same results (Table 7). With our media, however, deficiencies of biotin in the diet have a less drastic effect both on larval development and on eclosion. It is worth noting in this connexion that *Drosophila* needs far less biotin than *Aedes* (0·25 μg./5 ml.), according to Trager (1948) or *Tribolium* (0·5 μg./5 gm.) as determined by Fraenkel & Blewett (1943). Also, the lecithin present in medium B does not reduce the biotin requirement of *Drosophila* in the same way as it does that of *A. aegypti* (Trager, 1948). This suggests that biotin metabolism is different in the latter two species. There is also no evidence of an overdose effect such as that found by Fraenkel & Blewett (1943) with *Tribolium*.

Table 7. *Response to Biotin* (*medium B*)

Biotin per 5 ml. medium (μg.)	Mean larval period (log days)	*s*	*n*	% survival
Nil	0·870	0·044	15	18·7
0·005	0·852	0·028	43	35·8
0·010	0·831	0·042	47	39·1
0·020	0·814	0·033	32	80·1
0·040	0·779	0·054	98	81·5
0·080	0·741	0·030	50	63·0
0·160	0·742	0·036	62	77·5
0·320	0·743	0·026	68	85·0
0·640	0·735	0·026	28	70·0

VI. *Pyridoxine*. Vitamin B_6 was incorporated in Tatum's (1939) semi-synthetic medium apparently because it had been found necessary for the mosquito (Trager & Subbarow, 1938), and it has generally been used since. Hinton, Noyes & Ellis (1951), were unable to determine the dose response curve for this vitamin, although they showed that they got better growth with 15 μg. per 5 ml. of medium than with ten times this amount. We find that pyridoxine is essential for *Drosophila*, and that larvae fail to grow and die as early as second instar stages without it. Small amounts of the vitamin will allow some larvae to grow slowly and to pupate, but under these conditions only a few small flies emerge. As the pyridoxine is increased, a greater proportion of pupae form, but about 0·7 μg. per 5 ml. medium is needed to ensure that these all emerge (Fig. 12). The minimal supply for full growth is about 1·2 μg. per culture and up to 150 μg. per culture has no deleterious effect.

VII. *Folic acid* (*Pteroylglutamic acid*). Hinton (1952) has published dose-response curves for folic acid which show that no pupae can form unless 8 mμg. of this vitamin are included in the diet. Optimum growth requires at least 6 μg per 5 ml. of this medium, but 150 mμg. gives only slightly sub-optimal development and normal survival. Folic acid, therefore, appears to act as a larval growth stimulant and to be essential for pupation. Under our different conditions we have been unable to confirm these findings in detail. In the first place, larvae will grow and pupate on the casein medium in the absence of folic acid, even when the casein has been specially extracted to ensure that it is folic free (Table 8). But the pupae then formed generally fail to eclose although the unemerged adults are fully formed and on dissection appear normal except that their gonads are immature. This difference may derive from differences in the strains tested or from the use of media of different composition: the point has not been pursued further. Perhaps more important is our finding that as little as 1 μg folic acid is sufficient to allow normal growth and development in medium B and that more folic acid than this has no effect on

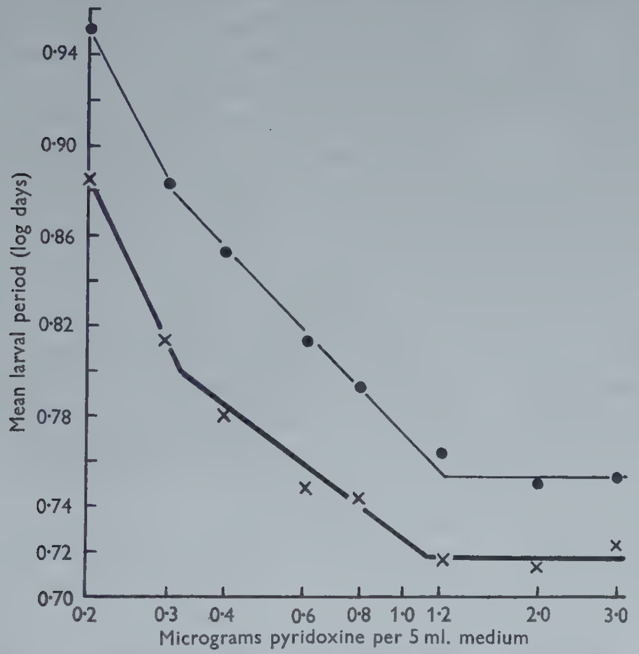

Fig. 12. Response to pyridoxine with medium A (upper curve) and medium B (lower curve). Only a small number of flies emerged at the lowest concentration shown.

Table 8. *Response to folic acid (medium B)*

Folic acid per 5 ml. medium (μg.)	Mean larval period (log days)	*s*	*n*	% survival	
				To pupa	To adult
Nil—extracted casein	—	—	nil	83	0
Nil—normal casein	0·841	0·024	5	89	4
0·25	0·784	0·044	96	86	80
0·50	0·769	0·040	100	83	83
0·75	0·747	0·031	102	86	85
1·00	0·737	0·033	107	91	89
1·50	0·741	0·043	110	93	92
2·00	0·755	0·040	95	80	79
3·00	0·727	0·033	100	86	83
4·00	0·740	0·039	94	79	78
8·00	0·736	0·030	102	87	85

development within the range tested. Our estimate of the minimal requirement of folic acid agrees with that used by Begg & Robertson (1950). If yeast nucleic acid is not incorporated in the diet more folic acid is needed to give normal growth and the minimum is then about 4μg./5 ml. of medium, but even under these conditions pupae will form without folic acid being supplied to them. Folic acid is therefore not essential for pupation, although it is for eclosion.

VIII. *Choline.* Begg & Robertson (1950) confirmed Schultz *et al.*'s (1946) conclusion that addition of choline to the diet improved the rate of larval development. Hinton, Noyes & Ellis (1951), on the other hand, state that larval development is normal in the absence of choline but pupation is then inhibited. The apparent contradiction here derives from differences in the basal media used, since Hinton, Noyes & Ellis (1951) included lecithin in theirs whereas the others did not. We have already noted that lecithin can replace choline and our results with a lecithin-free medium (Fig. 13) make it very clear that there is a definite optimum supply of choline; development is slowed when more or less than this amount (about 200μg./5 ml. medium) is incorporated in the food. Further, larvae cannot grow without choline and usually die after about 20 days in its absence. If only a small amount of choline is supplied (20μg./tube) many of the pupae which form are inviable. Within the range tested, excess choline slows development but has no other ill-effect. It is worth noting that very much more than the equivalent of the optimum amount of choline can be supplied as lecithin without any effect on development and that choline added to medium B (containing lecithin) in the quantities detailed in Fig. 13 gives no dose-response curve. Lecithin can therefore protect the organism from the effects of excess free choline and is the better form in which to supply this latter substance in the artificial diet.

IX. *Other vitamins and growth factors.* Inositol and *p*-amino-benzoic acid were included in Schultz *et al.*'s (1946) medium, but Hinton, Noyes & Ellis (1951) have since shown that the former has no growth function for *Drosophila* and that the latter slows growth. These findings have been confirmed. Hinton, Noyes & Ellis (1951) also demonstrated that carnitine and strepogenin (lipoic acid) were not required by Drosophila whereas vitamin B_{12} (cyanocobalamin) appeared to increase the numbers of pupae formed. Medium B contained about 0·0005μg. of B_{12}/5 ml., primarily as a contaminant of the casein, and additions of this vitamin so as to bring the level to that used by Hinton (1955) had no demonstrable effect on survival or on development.

The only other vitamin-like substance which has been shown to affect the growth rate of larvae is the 2% sodium bicarbonate extract of whole yeast described by Begg & Robertson (1950). A similar extract prepared from the water-soluble portion of autolysed yeast does indeed speed up larval development significantly (Table 9). As the fraction prepared according to Begg & Robertson's (1950) prescription is clearly complex and contains substances already supplied in the diet in optimal amounts (medium C was used) it is not surprising that excess slows development. The nature of the effective part of this fraction has not been examined further.

Table 9. *Effect of adding Begg and Robertson's yeast fraction to a complete medium*

Amount of yeast extract per tube (mg.)	0	35	70	140	280	560
Development time (log days)	0·764	0·678	0·666	0·660	0·628	0·715
s	0·080	0·061	0·053	0·085	0·032	0·039
n	93	67	84	76	79	76

The yeast fraction is here defined in terms of the quantity of Difco dehydrated yeast extract used in its preparation.

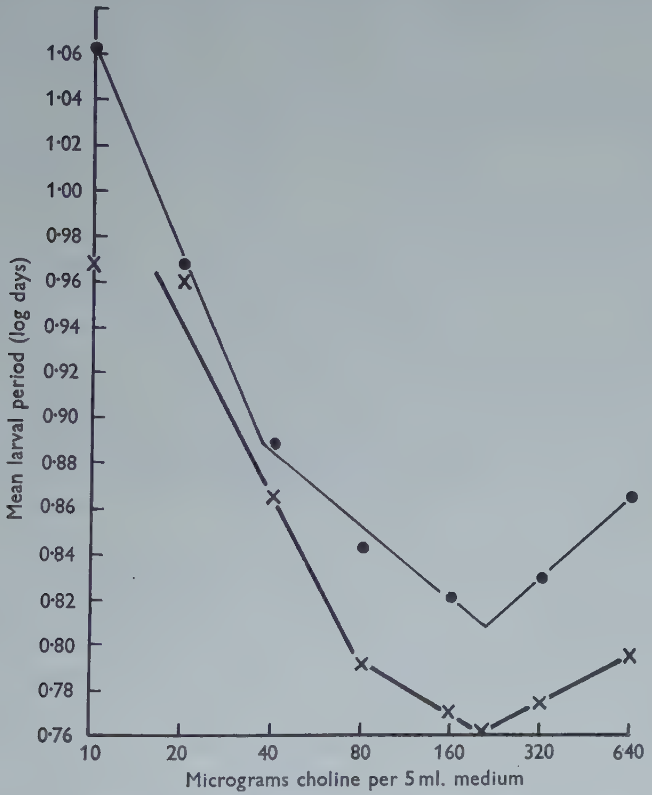

Fig. 13. Response to choline. Lecithin was necessarily omitted from the medium B shown in the lower curve. There is no survival when choline is left out of the diet.

Salt requirements. Trager (1947) states that 'the mineral nutrition of insects probably does not differ significantly from that of higher vertebrates'. While this may be so for some species, published data are still insufficient to support such a generalization, particularly with respect to *Drosophila* which seems to have exceptional mineral requirements. Loeb (1915) reared five successive generations of the fly on a yeast medium reputedly lacking Na and Ca salts, and Rubinstein, Lwowa & Burlakowa (1935) confirmed this finding by culturing larvae on yeasts which had been specially grown on media containing only traces of these elements. The resulting adults had about 5 % of the Na and 1 % of the Ca found in normal flies. It is therefore improbable that minimal requirements of salts correspond to the amounts found by conventional chemical analyses of adults.

Salt requirements could not be defined using our standard media since both the casein and agar were heavily contaminated with various metals, particularly calcium (1000 μg./5 ml.). None the less, it seemed worth simplifying the medium by finding if any of the salts usually incorporated could be eliminated from the diet. Tests showed that the Ca, Fe, Mn and Mg salts could be dispensed with under our conditions (Table 10), but the buffer could not be removed without causing the gel to break down when autoclaved.

Table 10. *The mineral requirements of* Drosophila *reared on ashless floc medium*

1. Salt added	Control	Nil	KH_2PO_4	$MgSO_4$+ KH_2PO_4	NaCl+ KH_2PO_4	NaCl+ $MgSO_4$+ KH_2PO_4
Development time (log days) No. of adults	0·894 19	∞ 0	∞ 0	∞ 0	∞ 0	0·871 49
2. Salt added	Control	Nil	$NaHCO_3$	$MgCO_3$+ $NaHCO_3$	$MgPO_4$+ $NaHCO_3$	$MgCO_3$+ KH_2PO_4+ $NaHCO_3$
Development time (log days) No. of adults	0·862 27	∞ 0	∞ 0	∞ 0	∞ 0	0·804 50

The salts were supplied so as to give a final medium concentration of 0·001 molar. Controls were as listed in Table 1 C. Additions of Ca, Fe and Mn separately and in combination gave no improvement over the responses shown above and are therefore not detailed here.

By replacing the standard casein with B.D.H. Ca-free casein and the agar with Whatman's ashless floc, a pulp medium was devised which helped to throw a little further light on this problem. Although the physical texture of this medium was much less favourable to early larval development it proved adequate for the purpose of checking the result just stated. Table 10 details the responses to various salts fed singly and in combination, and it is clear from these that the list of essential elements for *Drosophila* must include K, P, Mg and Na. For practical purposes only these elements need be included in the diet since the other trace requirements (see Kikkawa, Ogita & Fujito, 1955) will be met from contaminants in the remaining constituents of the diet. It is also worth emphasizing that this minimum list does not include Ca, which must be required only in trace amounts by *Drosophila*, whereas it is clearly essential for *Aedes* (Trager, 1953).

IV. DISCUSSION AND CONCLUSIONS

The results just detailed should allow the formulation of a synthetic culture medium as good as can be obtained within the limitations necessarily set by the need for handling food and larvae under aseptic conditions. Since the normal food of *Drosophila* is particulate (living yeasts and bacteria) an agar gel medium cannot be expected to give fully optimal growth, and the most reasonable comparison of the medium would be with an optimal supply of killed yeast in a similar gel. By this standard the complete synthetic medium is only just suboptimal (Table 11). Measurements of the growth rate of larvae clearly indicate that early growth is retarded when a gel medium is used and that this persists longer with the synthetic medium (Table 11). In other words, the first, and early second, instar larvae find it difficult to feed on a non-particulate surface and it is only when they are large enough to deal with the more homogeneous synthetic medium that they are able to develop normally. It is difficult to see how this difference can be eliminated; but for most purposes for which a synthetic medium is used it should be of little significance.

Table 11. *Rate of development on medium C and on yeast*

Food supplied	Mean larval period (log days)	*s*	*n*	Size of larvae in mm. after		
				48 hr.	72 hr.	90 hr.
Medium C	0·682	0·038	148	—	—	—
Medium C+yeast extract	0·643	0·047	153	2·27	4·32	5·26
10 % killed yeast in agar	0·611	0·062	123	2·04	4·66	5·81
Live yeast	0·595	0·059	149	2·73	4·67	5·15

It will also be obvious from the data presented above that the most generally useful diet formula is not one which limits the supply of every nutrient to the experimentally determined minimum. Greater amounts of most of the vitamins can be used without any detrimental effect, and advantage has been taken of this in formulating medium C (Table 1) in which about ten times the experimentally determined minimum is used. The other constituents all show more or less clearly defined optima and these have been adopted for medium C. Other strains of *D. melanogaster* are likely to differ to some degree in their requirements of these constituents, and one reason for setting out the dose-response curves in detail is that they indicate the range, and to some extent the priority, over which to test for such differences. Advantage has also been taken of the other findings to eliminate non-essential salts and vitamins from the diet. It should be possible to modify medium C so as to give results for other strains comparable with those enumerated in Table 11.

Estimates of minimal requirements give no direct measure of the quantities of each nutrient needed by a larva; they define only the relationships between particular requirements, and a strain with the same absolute needs, but which fed faster, for instance, would show regularly smaller minimal requirements and vice versa. So comparisons of the minimal supplies for species having very different feeding habits, such as that tabulated by Trager (1953), are of limited value. However, it is worth noting that the minimal requirements defined in the preceding section fall well below those given by Trager (1953) for *Aedes aegypti* and by Fraenkel *et al.* (1950) for *Tribolium confusum* and *Tenebrio molitor*, and provide no support for the thesis that there is an inverse relationship between size and vitamin requirements, such as Beerstecher (1950) has found to hold for vertebrates. Indeed, for the majority of vitamins the converse is true, as might be expected since there is a directly proportional relationship between insect size and respiration rate (Edwards, 1953). Minimal vitamin requirements appear to depend primarily on metabolic rate and not on absolute size.

One series of values which may be of use when comparing nutritional needs of insects is the amount of each constituent needed to produce one gram of viable pupae. And for this, it is necessary to have an estimate of the efficiency of food conversion for the species concerned. Chiang & Hodson (1950) give the only estimate of this conversion index for *Drosophila* when they state that larvae consume between three to five times their own weight of yeast. These values lie within the range found for the silkworm (Hiratsuka, 1950, quoted by Trager, 1953) and for *Blattela germanica* (McCay, 1938) and have been used to calculate the range of minimum requirements of vitamins necessary to produce one gram of *Drosophila* pupae set out in Table 12.

Table 12. *Estimated intake and content of the main vitamins—micrograms of vitamin per gram wet weight*

	B_1	B_2	Nicotinic	Pantothenic	Pyridoxine	Biotin	Folic	Inositol
D. Melanogaster pupae: intake	0·6–1·0	2·4–4·0	3·0–5·0	4·5–8·5	0·7–1·2	0·05–0·08	0·6–1·0	nil
content*	—	14·2	48·0	31·2	—	—	—	—
D. virilis larvae: content†	4·3	8·2	37·0	20·3	1·12	0·36	14·9	202
Brewer's yeast: content†	8·5	15·2	126·0	42·5	1·0	0·07	1·1	278

* Data from Charconnet-Harding & Calet (1951).

† Data from Williams (1943). Intake is estimated as lying within the range of three to five times the minim supply needed in 1 ml. of synthetic medium.

Comparison of the minimal requirements (Table 12) with available analyses of the vitamin content of *Drosophila* and of yeast is instructive. First, it is clear that the vitamin contents found by analysis of larvae or of pupae are primarily a reflexion of the amounts available in the yeast and are not a measure of minimal needs. There are apparently two exceptions to this rule: biotin and folic acid. In both cases the amount of vitamin found in the larvae greatly exceeds (by five and thirteen times respectively) the quantity present in the diet, whereas the larval content of the other vitamins is about half that found in brewer's yeast. This may imply selective storage of these two vitamins or, particularly in the case of folic acid, that the larvae have a limited ability to synthesize this vitamin, as our experimental data suggest (Tables 7 and 8). *Drosophila* larvae have, in fact, the highest folic content of all the species and tissues analysed by Williams (1943). Second, it is obvious that minimal requirements bear no constant relationship to the amounts of the different vitamins found in yeast although they tend to retain the same quantitative ranking with respect to each other. This divergence may be a consequence of the selection which has taken place during the breeding of the pure line of flies used in these experiments, or of differences in the culture conditions under which they are normally raised. So it cannot be said that requirements are not closely adapted to the normal nutritional supply (live yeast growing on cornmeal-molasses-agar) although the evidence of Table 12 is against such a conclusion. Put shortly, it seems that *Drosophila* larvae are able to grow normally on much less of these vitamins than would generally be available to them when they feed on an adequate supply of live yeast. This implies that growth is usually limited by some other circumstance, and Sang, McDonald & Gordon's (1949) data suggest that this is the general food shortage which occurs during the third to fifth days of development of a normal culture. The characteristics of the yeasts then available to the larvae are not known although they are apparently different from those of the yeast seeded on to the cultures (Sang *et al.* 1949), so it is impossible to judge if larval requirements are closely adapted to the yeasts present during this critical stage of culture growth.

Deficiencies of essential nutrients do not produce in *Drosophila* particular 'disease' syndromes of the kinds found with vertebrates. In some cases they result in death during the larval stage (aneurin, pantothenic acid, pyridoxine, etc.) and are often characterized by a high mortality during a particular instar, as we have noted. Shortage of other vitamins (nicotinic acid, folic acid) results in death during the developmental crisis of the pupal instar. But whenever adults emerge, their appearance is invariably normal, although their size may be reduced (benign melanotic tumours are frequently found under these stress conditions as Mittler (1952) has recorded). Indeed, the adaptability of larvae when confronted with dietary deficiencies is more surprising than the other effects of these shortages, as the data presented above bear out. This flexibility is, of course, an aspect of the ability of larvae to survive intense overcrowding (Sang, 1949) and is presumably an adaptation to the normal habit of developing in extremely limited, small breeding sites.

Since few organisms have been reared aseptically, it is impossible to say how far even their qualitative food requirements compare with those of *Drosophila*. Qualitative requirements of the few insects which have been studied seem very similar (see Trager (1953); Sedee (1953) for more recent work on *Calliphora*) but such work as has been done does not always distinguish between what is essential and what merely improves the growth of the species concerned. It is worth noting in this context that *Drosophila* larvae can grow without sugar, lecithin or RNA; without inositol (which they normally contain—Table 12) *p*-aminobenzoic acid, strepogenin, carnitine and possibly biotin, folic acid and cyanocobalamin. Although most of these substances are found in yeasts, the larvae have retained the ability to synthesize such of them as they need. It will be of considerable interest to learn if other insects with quite different feeding habits have also retained these abilities or if they show some other pattern of qualitative nutritional requirements.

V. SUMMARY

1. A technique for sterilizing large numbers of *Drosophila* eggs is described. This gives about 95% successful cultures.

2. Using this method, dose-response curves have been obtained for all the main dietary constituents under conditions in which interactions between them are likely

to be of little significance. Responses to the following are detailed: casein, fructose, cholesterol, lecithin, RNA, thiamine, riboflavine, nicotinic acid, pantothenic acid, biotin, pyridoxine, folic acid, choline and a water-soluble fraction of yeast which improves growth.

3. Other substances which have been included in *Drosophila* media are shown to have no value for the inbred strain of flies tested.

4. It is also shown that only K, P, Mg and Na salts need to be included in the food.

5. On the basis of these findings an optimal medium is formulated which allows larval development to be completed in about 4·4 days. This compares with 4·1 days when the same strain is reared on an optimal supply of killed yeast.

6. The minimal nutritional requirements of *Drosophila* larvae are shown to be considerably less than those of other aseptically cultured insects and, as far as the vitamins are concerned, only broadly related to the amounts found in yeast. The possible reasons for this are discussed.

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STUDIES ON THE RESPIRATION OF SEA-URCHIN SPERMATOZOA

I. THE EFFECT OF 2,4-DINITROPHENOL AND SODIUM AZIDE

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Humphrey (1950) has established by microspectroscopic examination the existence of cytochrome spectra in oyster spermatozoa, and has further shown that cytochrome inhibitors such as cyanide and azide suppress the oxygen uptake of these spermatozoa. In sea-urchin spermatozoa, however, Barron, Nelson & Ardao (1948*b*) have reported a contradictory result that, though the respiration is markedly depressed by cyanide, it is completely resistant to azide, even at high concentrations. On the other hand, Utida & Nanao (1954) have reported that the normal respiration of sea-urchin sperm is not affected by sodium azide, whereas the augmented respiration caused by 2,4-dinitrophenol (DNP) is completely abolished by this inhibitor. Since the presence of the cytochrome system in sperm cells has repeatedly been confirmed in a number of animals including sea urchins (Ball & Meyerhof, 1940; Zittle & Zitin, 1942; Mann, 1945; Rothschild, 1948), it is curious that azide fails to inhibit the normal respiration of sea-urchin spermatozoa.

Since Gray (1928) discovered that dilute sperm suspensions of sea urchin consume oxygen at a higher rate than dense ones, much work has been done to clarify the nature of this Dilution Effect (cf. Rothschild, 1951). Upon dilution of a dense suspension with sea water, there can be observed an increase in motility, and at the same time a sudden burst of respiration, which gradually subsides and settles to a steady level of oxygen uptake.

The present work has revealed that sodium azide exerts an inhibitory effect only on this initial burst. Consequently, during the subsequent course of low respiratory activity, the inhibitory effect is not noticeable. The effect of DNP on the Dilution Effect has also been investigated.

MATERIAL AND METHODS

The sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, were used. After sexing electrically (Iwata, 1950), semen was obtained in a clean syracuse dish by introducing isotonic KCl solution into the body cavity. The semen was then centrifuged for 5 min. at 3000 r.p.m. and the packed sperm thus obtained was diluted to desired densities with filtered sea water. Oxygen consumption was measured by the usual Warburg technique with flasks of about 20 and 10 ml. capacities. The gas phase contained air. The shaking rate was 100 c.p.m. with a 4 cm. stroke and the temperature was 20° C. All reagents were prepared afresh before each experiment and were adjusted to pH 8·2 if necessary.

For sperm counts a haemocytometer with Thoma ruling was used. It was shown that 1 ml. of the packed sperm contained about 5×10^{10} spermatozoa.

RESULTS

Initial burst of respiration. The Dilution Effect was examined in the spermatozoa of *Pseudocentrotus*. 1 ml. of dense suspension was put in the main chamber of the flasks and 0·5 ml. of sea water in the side arm. The time of mixing was taken as zero time ($t = 0$). The results are shown in Fig. 1, in which the respiratory activity is expressed in terms of Z_{O_2} (μl. O_2 per 10^8 spermatozoa per hour). The presence of the Dilution Effect is readily noticeable. From these curves it can be said that the initial burst is more rapidly lost in dense suspensions than in dilute ones. Further dilution beyond 1:50 does not increase the height of the curve, though it results in a prolongation of the initial high respiratory activity. After the initial burst is over, the respiration is maintained at a fairly constant rate for several hours. It is

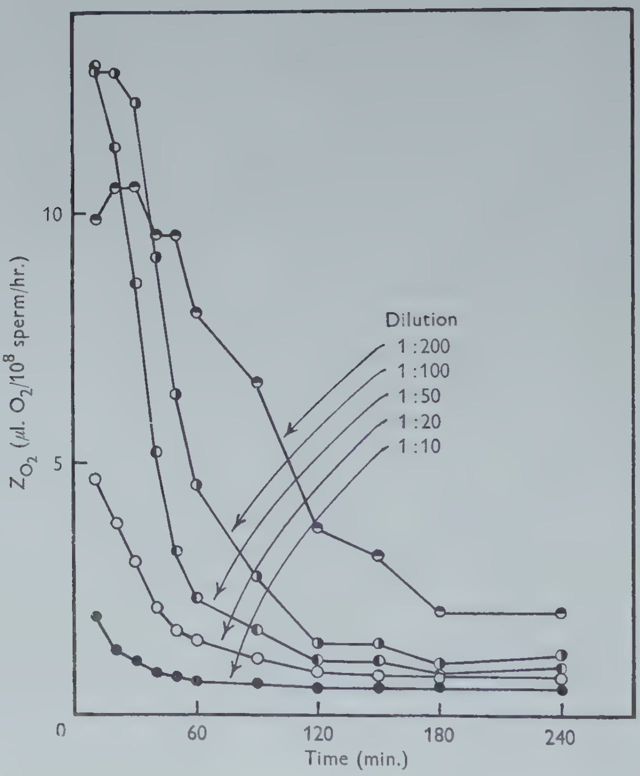

Fig. 1. Effect of dilution with sea water on the rate and the duration of initial O_2 uptake of sea-urchin spermatozoa (*Pseudocentrotus depressus*). 1 ml. of sperm suspension in the main chamber, 0·5 ml. of sea water in the side arm, tipped at $t = 0$. Temp., 20° C.

noteworthy that, in such a state, dense suspensions (especially in 1:10 and 1:20) still retain the capacity to exhibit another burst of respiration on further dilution, while in dilute suspensions (e.g. 1:200) this does not occur.

In addition, it was noted that in some cases, perhaps with poor material, the initial burst in dense suspensions failed to occur, the respiration proceeding at a steady low level from the beginning. The materials used by Utida & Nanao (1954) and by Barron *et al.* (1948*b*) must have been in such a state, as will be seen from the following sections.

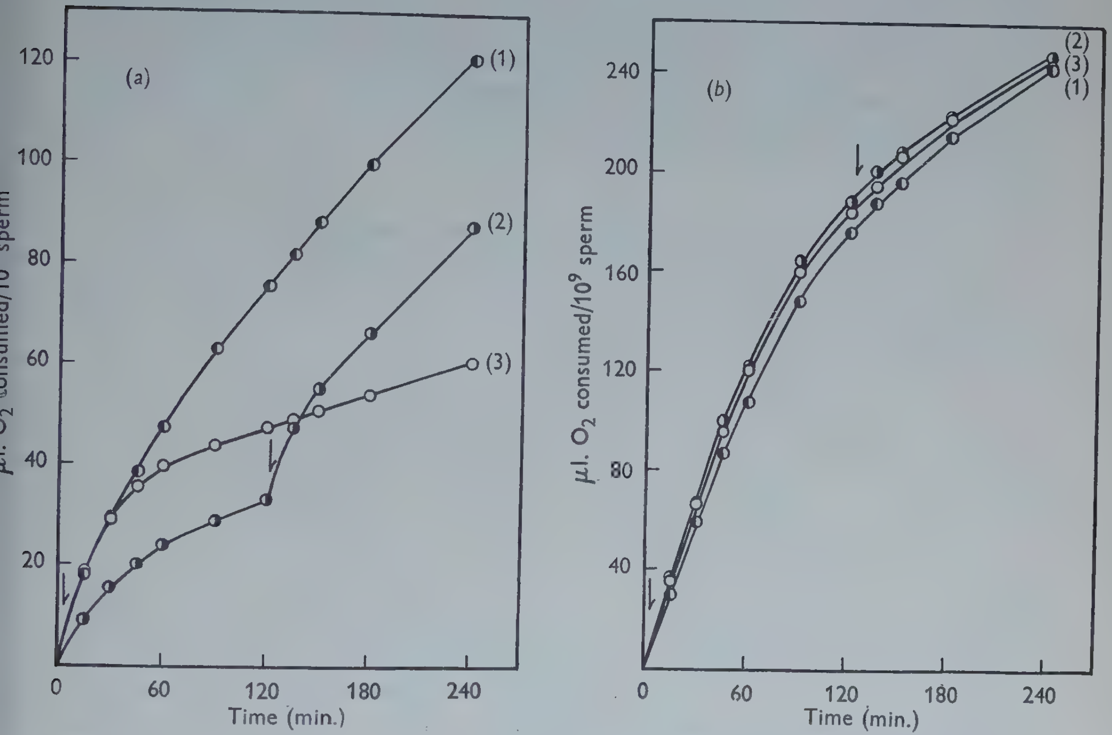

Fig. 2. Effect of DNP (1×10^{-4} M) on O_2 uptake of sea-urchin spermatozoa (*Pseudocentrotus depressus*). 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of sea water or DNP in the side arm, tipped at time indicated by arrows. (1), +DNP at $t=0$; (2), +DNP at $t=120$; (3), control. 20° C. *a*, dilution 1:20; *b*, dilution 1:200.

Effect of DNP on O_2 uptake of spermatozoa. The effect of DNP was examined in both dense (1:20) and dilute (1:200) sperm suspensions, using *Pseudocentrotus*. 1×10^{-4} M DNP, which was found to be optimal for increasing the oxygen uptake of the spermatozoa, was used throughout the experiments. Typical results with the dense suspension are shown in Fig. 2*a*, in which the curves represent the total oxygen consumed per unit quantity of spermatozoa. When DNP is added at $t=0$, during the first 30 min., no difference in the rate of oxygen uptake can be detected in comparison with the control. However, when the respiration of the control begins to come down to settle to the steady state, the difference becomes obvious and the DNP respiration remains high for several hours. The addition of DNP during the steady state at 120 min. results in an instantaneous increase of oxygen uptake. The addition of the same volume of sea water as that of DNP (one-tenth of the total volume) at $t=120$ brings about no significant changes in the rate of oxygen uptake (cf. Utida & Nanao, 1954). Thus, the steady-state respiration of dense suspension can be enhanced considerably by DNP, while the initial burst is not altered in the presence of this reagent. In the dilute suspension, on the other hand, DNP does not increase the oxygen uptake, whether it is added at $t=0$ or $t=120$ (Fig. 2*b*), although lack of effect at $t=120$ is attributed to the fact that the sperm is, by this time, in a poor condition. This result was repeatedly confirmed. In Table 1, the degree of the DNP effect during the steady state of respiration is compared among different dilutions. The stimulating action of DNP decreases inversely with the increase of dilution and becomes almost unnoticeable at the dilution of 1:200.

Table 1. *Effect of 2,4-dinitrophenol (DNP) on oxygen uptake of sea-urchin spermatozoa* (Pseudocentrotus depressus)

(Oxygen uptake was measured 120 min. after preparation of suspensions. The figures given are μl. O_2 per 10^9 spermatozoa per hour. Temperature, 20° C. DNP, 1×10^{-4} M.)

Dilution	O_2 uptake		% (control = 100)
	Control	+DNP	
1:10	3·0	13·5	450
1:20	4·5	16·1	358
1:50	10·4	28·0	269
1:100	18·7	25·0	134
1:200	23·1	24·9	108

The above would suggest that there is some respiratory system in spermatozoa, which comes into full activity when diluted with sea water, and that DNP acts upon such a system in a manner resembling the Dilution Effect. However, the oxygen uptake per unit quantity of spermatozoa is lower in dense than in dilute suspensions, even after the addition of DNP.

No attempt was made to estimate sperm motility quantitatively, although a rough observation showed that DNP slightly reduces motility despite its stimulating effect on respiration.

Effect of azide on O_2 uptake of spermatozoa. The results obtained with sodium azide in *Pseudocentrotus* (Table 2) and *Hemicentrotus* (Fig. 3) will be given. Sodium azide, if added at $t=0$, produces a marked inhibitory effect on respiration at concentrations of 1×10^{-2} M and 1×10^{-3} M. Even at lower concentrations such as 1×10^{-4} M and 1×10^{-5} M, a slight inhibition can be noted. This is consistent with the results of Humphrey (1950) on oyster spermatozoa. Sperm motility in the two sea urchins is completely abolished at 1×10^{-2} M of azide and affected slightly by 1×10^{-3} M, azide thus differing from cyanide which has little effect on the motility of sperm cells.

Table 2. *Effect of sodium azide on the respiration of sea-urchin spermatozoa* (Pseudocentrotus depressus)

(Flask volume, 10 ml. 1 ml. of suspension in the main chamber, 0·5 ml. of azide in sea water in the side arm, tipped at $t=0$. Temperature, 20° C.)

Dilution	Conc. of azide	$-\mu$l. O_2			
		1st hr.	2nd hr.	3rd hr.	4th hr.
1:20	0	154·3	68·5	44·8	43·3
	1×10^{-2} M	35·4	38·4	41·5	39·2
	1×10^{-3} M	78·3	42·4	37·4	39·5
1:200	0	21·2	11·4	5·3	6·1
	1×10^{-2} M	2·2	6·7	6·0	6·9
	1×10^{-3} M	8·0	6·4	5·6	6·8

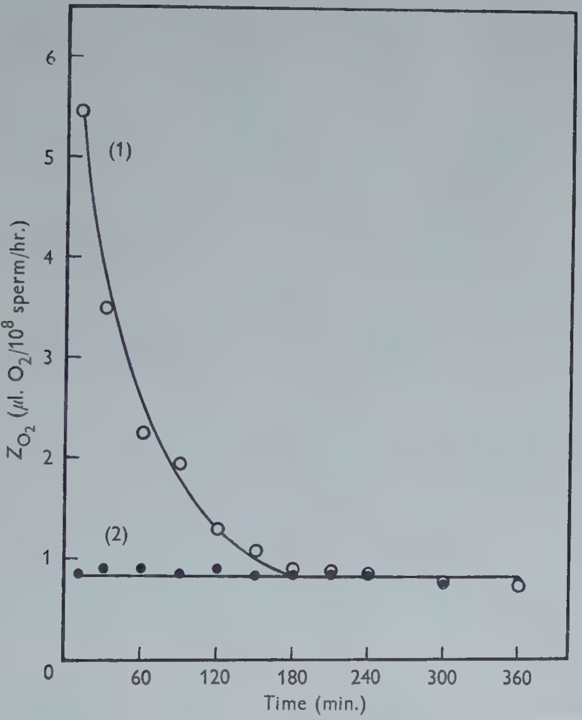

Fig. 3. Effect of sodium azide (1×10^{-2} M) on O_2 uptake of sea-urchin spermatozoa (*Hemicentrotus pulcherrimus*). 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of sea water or sodium azide in the side arm, tipped at $t=0$. Dilution 1:20. 20° C. (1), control; (2), +sodium azide.

Sodium azide, however, becomes non-effective in regard to oxygen uptake after 120 or 180 min. in both dense (1:20) and dilute (1:200) suspensions. This can be seen from Table 2 (compare the figures for 1st and 2nd hour against those for 3rd and 4th hour) and Fig. 3. In other words, the intense respiration associated with dilution is sensitive to sodium azide, but the subsequent lowered respiration is insensitive to it, as has been reported previously (Barron *et al.* 1948*b*; Utida &

Nanao, 1954). Furthermore, it can be noted that, in the presence of 1×10^{-2} M of azide, the inhibited level of respiration of the initial phase coincides with that of the later steady respiration. Consequently, from the standpoint of azide-action, it appears that two different components are at work in the respiration of spermatozoa, i.e. azide-sensitive and azide-insensitive ones, the initial burst consisting of both of them and the subsequent low respiration consisting of the latter alone.

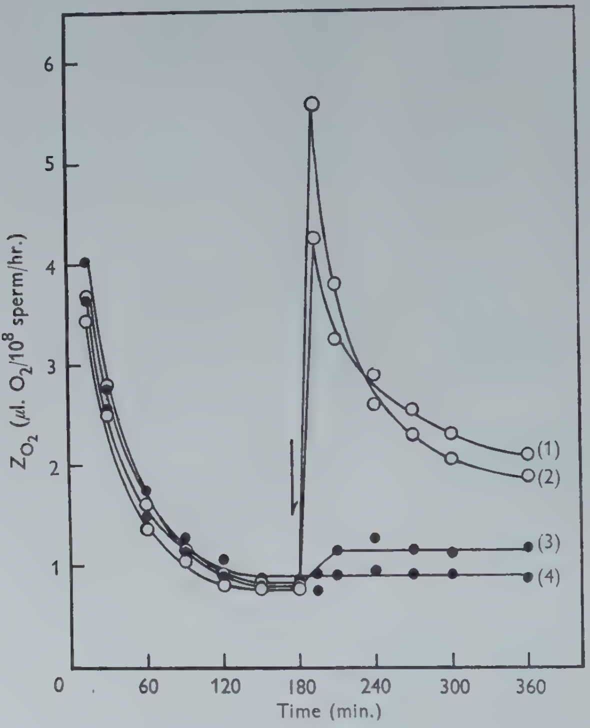

Fig. 4. Reversion from the azide-insensitive to the azide-sensitive respiration by the addition of DNP (1×10^{-4} M) and $ZnCl_2$ (1×10^{-6} M). *Hemicentrotus pulcherrimus*: 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of reagents in the side arm, tipped at $t = 180$. Dilution 1:20. 20° C. (1), +DNP; (2), +DNP+sodium azide (1×10^{-2} M); (3), $+ZnCl_2$; (4), $+ZnCl_2$ +sodium azide.

It has been mentioned that DNP can bring about an outburst of respiration if added to the low steady phase of dense suspensions. A similar effect was obtained by Rothschild & Tuft (1950) by the addition of trace metals such as copper and zinc, and the significance of the presence of these metals in natural sea water was suggested in relation to the Dilution Effect. Fig. 4 shows that the restored oxygen uptake after adding DNP and $ZnCl_2$ is also azide-sensitive. With DNP, however, the extra oxygen uptake is not completely eliminated by azide at 1×10^{-2} M, resulting in some rise in the level of the azide-insensitive residue. This is also seen from the experiment in which DNP is added at $t = 0$, as the following figures exemplify. For instance, figures of oxygen consumption in 1:20 suspension with and without DNP are 166·2 and 154·3 μl. respectively for the first hour, while those in the presence of azide (1×10^{-2} M) are 57·7 and 35·4 μl. This difference is maintained for several hours. In other words, the azide inhibition of DNP-augmented respiration is not so thorough as respiration augmented by dilution, indicating some modification in metabolic systems by DNP. With $ZnCl_2$, on the other hand, this has not been observed.

With potassium cyanide, another cytochrome oxidase inhibitor, the respiration of sea-urchin spermatozoa is almost completely inhibited at the concentrations of 1×10^{-4} M and 1×10^{-5} M (after Robbie's method, 1946). The azide-stable respiration in dense suspensions is also inhibited by this inhibitor.

DISCUSSION

The present experiments point out the necessity of paying more attention to the Dilution Effect in the analysis of the action of metabolic activators and inhibitors on the respiration of sea-urchin spermatozoa. DNP, for example, causes a four- to fivefold increase in oxygen uptake of the steady state at a dilution of 1:10, but not at a dilution of 1:200. In the experiments using nitrogen mustards, Barron, Seegmiller, Mendes & Narahara (1948*a*) have reported some difference in the mode of action of these substances according to the sperm density.

Furthermore, the present results indicate that azide differs in its effect on the initial burst and on the subsequent steady respiration. Much evidence is available to show that 'activity' respiration is more sensitive to azide than 'resting' respiration (frog muscle, Stannard (1939); sea-urchin egg, Fischer, Henry & Low (1944); nerve tissue, Gerard & Dotty (1950)). It is, therefore, of interest that the initial burst seems to act like 'activity' respiration, and the steady-state respiration seems to correspond to 'resting' respiration. This is true in both dense and dilute suspensions, but of special importance is the fact that the azide-stable respiration of a dense suspension, which continues for a long time if left undiluted, can be changed reversibly to the azide-sensitive one on further dilution.

No difference was obtained in the respiratory quotient (R.Q.) of the initial and the subsequent respiration measured by the Warburg direct method, both having the values characteristic of carbohydrate metabolism (0·91–1·03).

Recently it has been reported that DNP also causes an increase in oxygen uptake, which can be abolished by azide in such a low concentration as to be ineffective on normal respiration (grasshopper eggs, Bodine (1950); sea-urchin eggs and sperm, Utida & Nanao (1954); toad embryos, Ishida, Uramoto & Maruyama, unpublished; etc.), and it is known that one of the actions of DNP is to convert the mitochondrial apyrase from a latent to an apparent state (cf. Lardy & Wellman, 1953). Maruyama (1954) has demonstrated in the toad embryo that azide almost eliminates the activity of the apparent apyrase (with DNP), leaving the latent apyrase activity (without DNP) unaffected. There is a possibility, therefore, that some enzyme system essential for the respiration and motility of sea-urchin spermatozoa is in a latent state before dilution and is converted into an apparent state on dilution.

Aside from such a latent-apparent mechanism, azide is a powerful inhibitor of cytochrome oxidase *in vitro*. Therefore it is quite probable that the cytochrome-cytochrome oxidase system begins to work on dilution. This proposition has been investigated, and the results will be presented in the next paper.

SUMMARY

1. The effects of 2,4-dinitrophenol (DNP) and sodium azide on the respiration of sea-urchin spermatozoa (*Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*) have been investigated in relation to dilution.

2. The initial outburst of respiration following dilution is higher in its rate and longer in its duration the more dilute the sperm suspensions are. After the initial high respiration has diminished, oxygen uptake in dense suspensions continues at a very constant rate for a long period of time, during which spermatozoa remain motile. A high level of respiration can be restored by further diluting a suspension in the steady state. In dilute suspensions, however, once the respiration has dropped, it can never be reactivated.

3. DNP (1×10^{-4} M) causes a marked rise in oxygen uptake when given during the steady state, but has little effect during initial burst.

4. Conversely, only the initial high respiration is inhibited by sodium azide, while the subsequent steady respiration is not affected by this poison. This is especially the case in dense suspensions.

5. When the initial high respiration is inhibited by azide at 1×10^{-2} M, the reduced level always coincides with that of the steady-state respiration. The respiration of sea-urchin spermatozoa thus seems to be separable into two components, one azide-sensitive and the other azide-insensitive.

6. The extra increase in oxygen uptake over the steady-state level on adding DNP or Zn in dense suspensions is also ascribed to an increment in the azide sensitive component.

7. Potassium cyanide inhibits not only the initial increase but also the steady-state respiration.

The author wishes to express his hearty thanks to Dr J. Ishida of Tokyo University for his warm encouragement during the course of this work. Thanks are also due to Dr K. Dan of Tokyo Metropolitan University for his kind guidance in the preparation of the manuscript.

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THE EPICUTICULAR WAX LAYERS OF THE PUPA OF *TENEBRIO MOLITOR* L.

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(With Plates 1 and 2)

INTRODUCTION

During the past ten years the structure of the outer epicuticle of insects has been studied in detail in a number of species. In all these examples there is a basal epicuticular layer of lipoprotein (cuticulin) of the order of 1 μ thick and from one to three outer layers, none of which normally exceeds 0·5 μ in thickness. In the bug *Rhodnius* (Wigglesworth, 1947) the surface of the cuticulin layer is covered by a layer rich in polyphenols; this layer is in turn covered by a wax layer secreted just before moulting. After moulting, a 'cement layer' is secreted over the wax, serving to protect it from solvents and abrasive dusts. A similar sequence of lipoprotein, polyphenol, wax, and cement layers has been described from the adult *Tenebrio molitor* (Wigglesworth, 1948*a*), the larva of *Diataraxia oleracea* (Way, 1950), and the tick *Ornithodorus moubata* (Lees, 1947). In the Diptera, however, there is no cement layer, the wax being exposed on the cuticle surface (Dennell, 1946; Wolfe, 1954), and a similar condition may exist in the Hymenoptera (Glynne Jones, 1955).

Despite these variations, the epicuticles of insects clearly conform to a single plan (Wigglesworth, 1948*b*; Dennell & Malek, 1953). The possession of a wax layer near the surface is a general characteristic of the group, and it is to this wax layer that the low permeability to water of the cuticle is due (Ramsay, 1935; Wigglesworth, 1945; Beament, 1945). In all the species of which a detailed study has been made, the wax layer is found to occur in the same morphological position. It lies above the cuticulin layer and below the cement, and where (as in the Diptera) a wax layer is exposed on the surface, the cement layer is absent. However, wax 'blooms' are known to occur on the surfaces of a number of species (Stellwaag-Kittler, 1954; Weber, 1934) and it may be questioned whether or not the cement layer is likely to be deficient in all these examples. In *Rhodnius* also, there is a hint that the fed insect may secrete wax on to its surface prior to the withdrawal of the pore canal contents (Wigglesworth, 1945). If this is the case, it would mean that a wax bloom is formed on the surface of a cement layer of normal type. Clearly, it is important that a detailed study be made of a surface wax bloom in order to determine its relationship to other cuticular layers. The present paper is concerned with the description of such a layer in the pupa of *Tenebrio molitor*, and evidence will be presented to show that this surface wax overlies a cement of normal type, below which there is a 'primary' wax layer comparable with that in other insects.

The discovery of two distinct wax layers in the *Tenebrio* epicuticle led to an investigation of their relative roles in waterproofing. The results of this investigation have necessitated a re-interpretation of the meaning of the 'critical temperatures' of insect water-loss/temperature curves.

In this study use has been made of two electron optical techniques, reflexion electron microscopy and electron diffraction, which have not hitherto been employed to any great extent in entomological problems. A brief account of the salient features of both techniques will, therefore, be given before the results obtained from *Tenebrio* pupae are discussed.

The reflexion electron microscope

It is possible to examine the surface of a solid object in the electron microscope directly. The method is illustrated schematically in Text-fig. 1. A beam of electrons is incident at a small angle (~ 1–2°) to the surface. The electrons are scattered by the object and the surface is viewed at a rather larger angle (~ 6–10°). Full accounts of this technique (usually known as 'reflexion' electron microscopy) are given by Menter (1952, 1953), but the main features of the method can be summarized as follows:

(1) The image is highly foreshortened, owing to the oblique angle of viewing. The magnification is therefore different in different directions in the image. Two principal magnifications, $m_{\parallel}$ and $m_{\perp}$, referring to directions parallel and perpendicular to the foreshortened direction can be quoted.

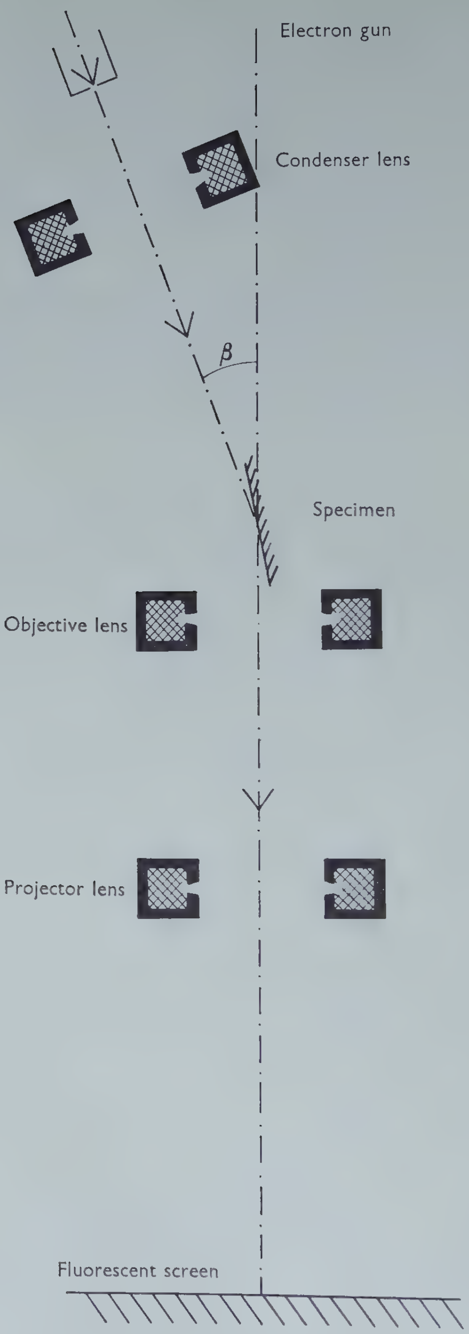
(2) There is a resolution in the image of 300–400 Å. in the direction of maximum magnification, and a resolution of 2000–4000 Å. in the foreshortened direction at right angles.

(3) Contrast in the image arises primarily from shadows cast by asperities. Owing to the oblique angle of illumination these shadows may be some thirty to sixty times as long as the asperities are high. Consequently, there is a very high contrast for small vertical features on a surface, and steps as small as 30 Å. in height can be detected on a suitable specimen.

(4) There is a large extent of focus in three directions at right angles.

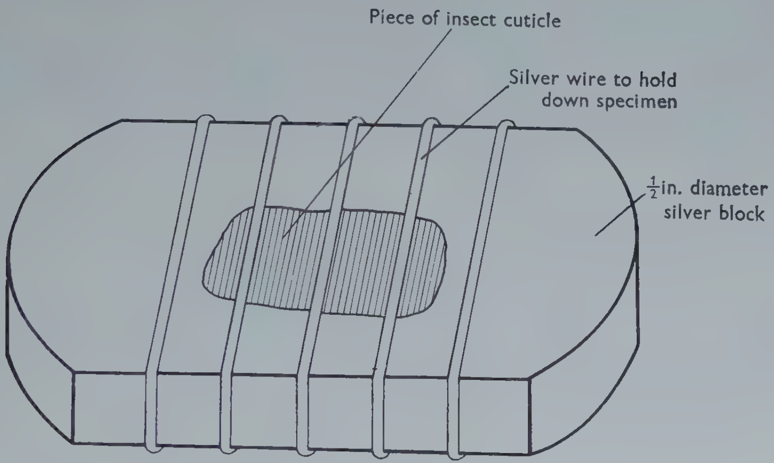
The instrument used in the present investigation was the Metropolitan Vickers EM 3 electron microscope modified for use in reflexion as described by Menter (1952, 1953) and subsequently by Courtney-Pratt, Menter & Seal (1956).

Specimens of the cuticle of *Tenebrio molitor* pupae for examination in the reflexion electron microscope were derived from animals which had pupated in clean glass specimen tubes. The animals were opened by a transverse cut across the dorsal cuticle, and the prothoracic cuticle freed by cutting around its margins. The lower surface of the cuticle was wiped with points of damp filter-paper to remove adhering cellular material, and was then mounted flat on a silver block. The cuticle was held



Text-fig. 1. Schematic representation of the reflexion electron microscope, showing the disposition of the illuminating system, electron lenses, and specimen.

down by silver wires as shown in Text-fig. 2. A film of silver about 300 Å. thick was then deposited of the surface by evaporation in vacuum. This is necessary to prevent charging of the surface in the electron beam during the electron microscope examination. The presence of the silver film should not have any detrimental effect on the resolution. Fuller details of technique have been given in a preliminary paper on the epicuticular structure of the *Tenebrio* pupa by Holdgate, Menter & Seal (1956).


Piece of insect cuticle
Silver wire to hold down specimen
$\frac{1}{2}$in. diameter silver block

Text-fig. 2. Schematic diagram showing the method of mounting insect cuticle for examination in the reflexion electron microscope.

Electron diffraction

In electron diffraction (see e.g. Thomson & Cochrane, 1939), the periodic atomic lattices of crystalline materials act as gratings for fast electrons and thus give rise to diffraction patterns. However, the penetrating power of electrons into matter is small and, consequently, one can only use very thin specimens in transmission or examine the immediate surface of a specimen in reflexion. The technique is thus very suitable for the examination of thin films and surface layers, and it is often possible to determine the unit cell dimensions of substances in such layers. In the present study, a Finch electron diffraction camera operated at about 50 kV. has been used to obtain diffraction patterns from epicuticular waxes, and it has been found possible to determine some of the cell dimensions of these waxes and so obtain information about their nature. The cell dimensions have been measured at temperatures between room temperature and the melting-point of the wax, using a small electrical heater to warm the specimen while it is being examined in the diffraction camera. This heater was designed by Menter & Sanders (1950), and the authors are indebted to them for permission to use it.

Material

The *T. molitor* pupae used in this work were derived from a laboratory stock cultured on bran and kept at room temperature (about 20° C.). Animals in culture are liable to become contaminated by the medium (Holdgate, 1955 *a*) and are thus unsuitable for study of their surface structure. Larvae were therefore removed from culture while in the immobile prepupal stage, transferred to clean glass specimen tubes, and kept until pupation in an incubator at 25° C. In this way uncontaminated specimens were obtained which showed consistent surface structure and wetting properties. The maintenance of a constant temperature in the incubator caused the animals to develop at a uniform rate, so that repeatable data could be obtained for the changes in surface structure with age.

In the reflexion electron microscope studies, the prothoracic cuticle was always chosen as the region for examination. Its selection was due to its size and flatness, ease of handling, and its general immunity from contact with surfaces on which the pupa was lying.

THE EPICUTICULAR WAX LAYERS IN THE *TENEBRIO* PUPA

The appearance and general properties of the pupa

Wigglesworth (1948 *a*), in his description of the deposition of the cuticle in the adult *T. molitor*, comments briefly on the appearance of the pupal cuticle. His observations refer chiefly to the contrast between the shiny-surfaced, white, young pupa and the dull-surfaced, pale brown, mature stage.

When first formed the pupa is white, and its surface is shiny in appearance. The cuticle in some regions, including the prothorax, is thrown into a series of shallow corrugations, and bears a few small bristles. Apart from these features it appears optically smooth. At moulting there are two prominent depressions on the prothorax; these disappear about 4 hr. afterwards (in specimens kept at 25° C.).

At about 10–12 hr. the cuticle darkens to a pale straw colour, but the surface remains shiny until about 14 hr. From then until 30 hr. it becomes progressively dulled, and after 30 hr. the surface is no longer glossy, but matt in appearance. There are no further visible changes in the pupal cuticle.

Surface structure in the newly moulted pupa

Apart from the shallow corrugations to which reference has been made above, the prothoracic cuticle of the newly moulted *Tenebrio* pupa appears optically to be nearly smooth. However, reflexion electron micrographs (Pl. 1 *a*) reveal a small-scale roughness in addition to the gross ridging. The micrograph shows only a part of one of the large ridges.

The small-scale roughness visible in Pl. 1 (*a*) is due to shallow wrinkling only, and is not inconsistent with optical smoothness of the surface. The water contact angles of the newly moulted pupal prothorax (Holdgate, 1955 *a*) have been found to be 112° (advancing) and 100° (retreating). Very little trapped air is visible when the cuticle is immersed in water, and this and the low hysteresis of the contact angle also indicate that the surface is fairly smooth. The true contact angles of the surface are certainly near to or above 90°, and this suggests that the cuticle surface is composed chiefly of hydrophobic —CH_3 groupings or others with similar water-repellent properties.

The newly moulted *Tenebrio* pupa is resistant to desiccation, the mean rate of water-loss in dry air at 25° C. being about 0·05 mg./cm.^{2}/hr. This figure was obtained using the method of Wigglesworth (1945), phosphorus pentoxide being the desiccating agent. The animals do not blacken when immersed in ammoniacal silver nitrate solution (Wigglesworth, 1945).

The resistance to desiccation is destroyed by washing pupae in chloroform or benzene in the cold for 2 min. After this treatment pupae show a general blackening when they are immersed in ammoniacal silver nitrate. Hand-cut sections show this blackening to be largely confined to the outer regions of the cuticle.

These observations are in close agreement with those of Wigglesworth (1947) on the newly moulted *Rhodnius* nymph. They suggest that in the young *Tenebrio* pupa a waterproofing wax layer covers the surface of the cuticle, and that this layer is exposed to the action of solvents because it lacks a cement covering. The extensive surface blackening which follows the removal of the wax layer suggests that a 'polyphenol' layer comparable to that of *Rhodnius* exists beneath the wax at this stage in cuticle formation. The wax layer appears to be morphologically homologous with that described from *Rhodnius*, from *Tenebrio* adults (Wigglesworth, 1948*a*), and from *Diataraxia* (Way, 1950), and many ticks (Lees, 1947), and will be termed the 'primary wax layer'.

The composition of the primary wax layer

While the solute properties of the primary layer described above suggest strongly that it is a 'wax' of some kind, they are far from giving a precise indication of its composition. On evaporation, benzene and chloroform washings of newly moulted pupae yield a white residue showing a rather indefinite melting-point of about 40° C. Twenty newly moulted pupae, washed in benzene at room temperature for 5 min., yielded 0·4 mg. of solid material. Assuming that all this was wax, and that all the material of the primary layer had been removed, the thickness of the primary layer can be calculated to be 0·15 μ. This is of the same order of magnitude as the 0·25 μ which Beament (1945) considered to be the typical thickness of insect wax layers.

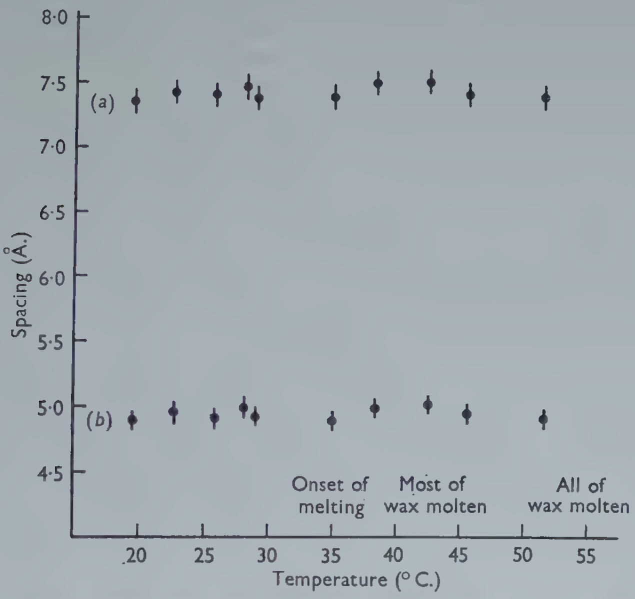
The residue obtained from chloroform washings of newly moulted pupae has been further studied by transmission electron diffraction. A good ring pattern, similar to Pl. 2 (*c*), was obtained, and from this the *a* and *b* unit cell dimensions of the material can be calculated. The pattern is typical of an orthorhombic, straight-chain, hydrocarbon compound with $a = 7{\cdot}36 \pm 0{\cdot}10$ Å., and $b = 4{\cdot}89 \pm 0{\cdot}07$ Å. Neither the *c* dimension, which is related to the length of the hydrocarbon chain, nor the nature of the end group of the chain can be determined by electron diffraction. It is, however, possible to use tilted transmission specimens to confirm that the hydrocarbon chains are normal to the collodion support film on which the wax is deposited from the chloroform solution. The waxes obtained from solution thus take up a regular orientation (cf. Beament, 1945). Such tilted specimens also show that there is a repeat distance in the hydrocarbon chain of approximately 2·5 Å. These values can be compared with those quoted by Wyckoff (1953) for long straight chain hydrocarbon compounds; $a=7{\cdot}45$ Å.; $b=4{\cdot}96$ Å.; C—C distance in the hydrocarbon chain $=2{\cdot}54$ Å.

An attempt was made to obtain a diffraction pattern directly from the surface of a young pupa, but without success owing to the charging of the specimen in the electron beam.

Measurements of the cell dimensions of the wax obtained from chloroform washings of young pupae were also made at temperatures up to the melting-point of the wax. By this method it was possible to test directly the hypothesis of Beament (1945) that insect waxes undergo 'crystalline transitions' at temperatures several degrees below their melting-points. Beament based his hypothesis on the fact that crystals of a given insect wax showed optical changes (usually increased transparency) at a temperature well below that at which the crystals as a whole were observed to melt. At this same temperature the wax crystals would show 'spreading' in a water surface and appeared to become more hydrophilic. Beament suggested that these changes were due to a transition of the kind which Müller (1930, 1932) observed with certain pure paraffins using X-ray diffraction. A transition of this kind occurs at a temperature several degrees below the melting-point, and involves an abrupt increase in the a dimension of the unit cell, and a smaller but equally abrupt decrease in the b dimension. The change in the packing of the chains in this transition was invoked by Beament to explain an apparent abrupt increase in the permeability of insect wax layers at a temperature comparable to that at which the extracted waxes showed optical changes. Now Müller's work was carried out on pure paraffins, while Beament extended it to insect waxes which are very far from being pure paraffins—they are mixtures of paraffins, alcohols, fatty acids, and esters of variable chain length (Chibnall, Piper, Pollard, Williams & Sahai, 1934). It seemed desirable, therefore, that some check should be made on whether these complex insect wax mixtures do in fact show any abrupt changes in the a and b dimensions of the unit cell at temperatures below the melting-point, and the incorporation of the electrical heater in the diffraction camera allowed this to be done.

The results of such an investigation on a wax sample from chloroform washings of newly moulted *Tenebrio* pupae are shown in Text-fig. 3. No significant change in the cell dimensions could be detected at temperatures up to the melting-point. However, the melting of the wax in a particular region of the specimen could be detected by the disappearance of the ring pattern of the solid, leaving only a diffuse ring of spacing 4·6 Å. due to hydrocarbon material in the liquid phase (Stewart & Morrow, 1927; Stewart & Skinner, 1928; Stewart, 1928; Morrow, 1928). The melting, which was by no means sharp, commenced at about 35° C. and was practically complete by 42° C., though a few isolated regions remained solid up to 55° C.

It would appear from these results that, in *Tenebrio* primary wax at least, there is no 'transition' in the sense of Müller (1930, 1932). *Tenebrio* pupal wax was not one of those studied by Beament (1945) and so this result does not directly conflict with his findings. But, the absence of a transition in this insect wax, taken together with the very wide range of melting-points in the one sample, might suggest another possible explanation of the optical changes observed in insect wax crystals by Beament at temperatures below the melting-point of the crystals as a whole. It might be that these optical changes were due to the melting of one or more components of a mixed crystal mass—the flow of the liquid wax along the interfaces between the crystals of higher melting-point thus increasing the transparency.



Text-fig. 3. Plot of *a* and *b* unit cell dimensions against temperature, for wax obtained from chloroform washings of newly moulted *Tenebrio* pupae.

The cement layer

The depressions on the prothorax disappear at about 4 hr. after pupation (at 25° C.), and the surface of the animal becomes even more shiny than at first. By about this stage it is found that blackening in ammoniacal silver nitrate no longer follows brief washing in wax solvents. By about 12 hr. practically none of the surface blackens when treated in this way, although insects immersed in boiling chloroform for 5 min. still show general darkening.

These observations agree closely with Wigglesworth's (1947) findings in *Rhodnius*, and seem to indicate that a cement layer is secreted in the *Tenebrio* pupa between 4 and 12 hr. During this period there is a slight fall in the contact angles against water, to values of about 100° (advancing) and 87° (retreating), but there is never a 'spreading stage' as in *Rhodnius* (Wigglesworth, 1947; Holdgate, 1955*a*).

Reflexion electron micrographs of the pupa at the 12 hr. stage do not show any significant differences from those of the newly moulted pupa.

Surface structure in the mature pupa

At about 14 hr. after the cuticle has darkened, there is a change in the appearance of the pupal surface. It ceases to be shiny, and from then until 30 hr. becomes progressively dulled, until the uniform matt appearance of the mature pupa is attained.

The surface structure responsible for this dulling is not rigid, and if a needle is passed over the cuticle a shiny trail is left in its wake. If the pupa is washed for 1 min. in cold chloroform or benzene the surface is rendered shiny, and the same thing happens if it is heated to above 80° C. These observations suggest that the dulling of the surface may be due to the secretion of a rough surface wax 'bloom' which is soluble in cold chloroform or benzene, and which melts at about 80° C., to form a smooth film.

The changes in contact angle against water between 14 and 30 hr. support this view (Holdgate, 1955*a*). Whereas at 14 hr. the shiny surfaced pupa has an advancing angle of 100° and a retreating angle of about 90°, by 30 hr. these have increased to 155° and 110° respectively. This increase is almost certainly due to increased surface roughness, and not to any change in the composition of the surface. After washing the pupa in chloroform or benzene for 1 min., the contact angles are reduced to 110° and 95° as in the young pupa. Heating to 80° or above produces a similar fall in the angles (which are not measured until the animal has cooled), presumably because of the melting of the surface material to form a smooth layer.

Further information about the structure of this rough surface layer has been obtained by examination of specimens of the mature pupal cuticle in the reflexion electron microscope. Pl. 1 (*b*) shows the appearance of the prothorax of such a mature pupa. The surface is now covered with a large number of fine filamentous projections. These are shown more clearly in Pl. 1 (*c*) which was taken with a lower angle β (Text-fig. 1). They are $0{\cdot}5$–$1\,\mu$ high, about $0{\cdot}1\,\mu$ wide and number 10^6 to 10^7 filaments per mm.^2. It is only possible to give an approximate estimate of the filament thickness because of uncertainties in the thickness of the evaporated silver deposit on the sides of the filaments, and because the details of the scattering of electrons at the edges of such projections are not known.

The filaments shown in Pl. 1 (*b*) and (*c*) provide an obvious explanation of the high surface roughness, which the contact angle measurements indicated, and confirm that the mature pupa has a surface layer which is not present in the newly moulted animal. The filaments are not visible in micrographs of mature cuticles, which have been washed with chloroform or heated to above 80° C. prior to examination in the reflexion electron microscope (Pl. 2*a*, *b*).

The secretion of the surface filaments is, at least to some degree, an active process; it does not depend on a passive change in layers already present at pupation. The surface layer appears between 14 and 30 hr. in animals kept at 25° C.; in animals kept at 17° C. it does not appear until about 30–40 hr., while animals kept in a cold room at 1° C. remain shiny for several weeks. But, if the surface layer is removed from mature pupae by brief washing with a wax solvent it is not replaced. The animals thus treated continue to develop, moulting to adult after the normal period of 8 days. They generally fail, however, to cast the pupal cuticle. Mature pupae which have been washed in cold chloroform will not blacken on immersion in ammoniacal silver nitrate, and remain resistant to desiccation. It appears, therefore, that the cement layer is still present and that this protects the animal against complete removal of its waterproofing waxes by the cold solvents. Treatment with boiling chloroform destroys the resistance to desiccation and causes a general blackening on subsequent immersion in ammoniacal silver nitrate.

The composition of the surface layer

The only evidence so far presented which suggests that the surface filaments are composed of wax is their solubility in chloroform and benzene, their apparent fusion by heat, and their large water contact angles. As with the wax of the primary layer, further evidence has been obtained by the study of the material deposited from cold chloroform and benzene washings.

This material is a white, semi-crystalline solid. Fifty pupae washed in benzene for 2 min. yielded 5·1 mg. of residue which, assuming that it was all wax, would be adequate to form a layer 0·75 μ thick over the surface of each animal. This figure can be compared with the filament length of 0·5–1 μ obtained from the reflexion electron micrographs, but it must be remembered that there are gaps between the filaments.

Evidence as to the nature of the material was obtained by electron diffraction examination of the residues deposited from cold chloroform washings of mature pupae. The substance gives a good ring pattern (Pl. 2*c*), and the spacings of these rings are given in Table 1. It was found possible in this case to examine a mature pupa directly in the electron diffraction camera, and a similar ring pattern was obtained. The spacings of the rings in this pattern are also shown in Table 1, and it can be seen that the pattern given by the surface layer *in situ* is closely similar to that given by the residue laid down from chloroform washings. This provides valuable confirmation that the surface filaments are in fact of the same crystallographic nature as the material obtained from the chloroform washings.

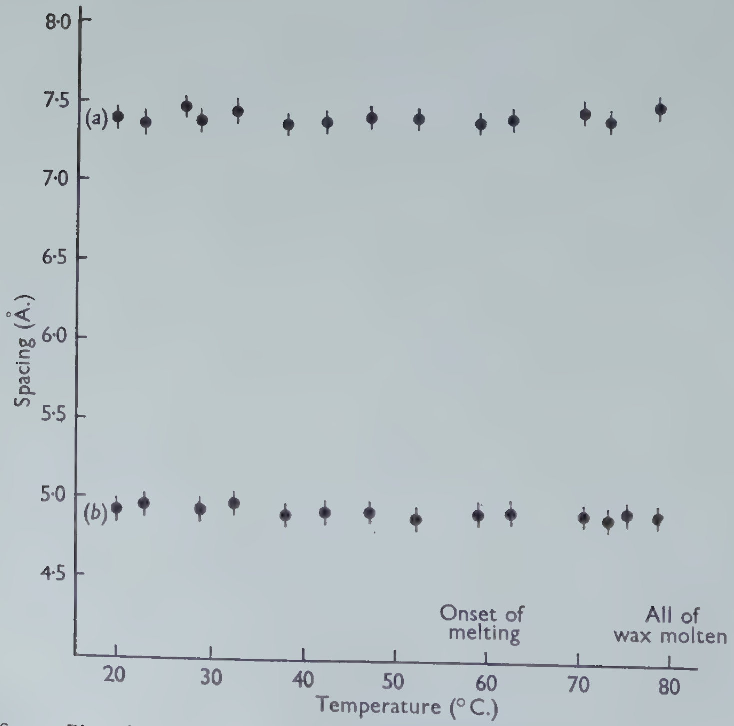
The patterns are again typical of an orthorhombic straight chain hydrocarbon compound ($a = 7{\cdot}44 \pm 0{\cdot}02$ Å.; $b = 4{\cdot}97 \pm 0{\cdot}01$ Å.; C—C distance (tilted specimen) $= 2{\cdot}47 \pm 0{\cdot}05$ Å.). It is again emphasized that it is not possible to obtain evidence as to the length of the hydrocarbon chain or the nature of the end group by electron diffraction.

The cell dimensions of the surface wax (as deposited from chloroform solutions) were measured at a series of temperatures up to its melting-point, and the results are shown in Text-fig. 4. The wax began to melt just below 60° C., and all parts of the specimen were liquid by 82° C. There was no evidence of any abrupt changes in the *a* and *b* dimensions at temperatures below the melting-point.

The melting-points of samples of this material were also determined optically using a standard procedure. The wax, in a sealed glass capillary tube, was attached to the bulb of a mercury-in-glass thermometer and warmed in an oil bath. The sample was observed microscopically, and any visible changes in the sample were noted, together with the temperatures at which they occurred.

Table 1. *Bragg spacings obtained from electron diffraction examination of: (a) the residue left on evaporation of chloroform washings of mature* Tenebrio *pupae, (b) the surface of a mature* Tenebrio *pupa*

(*a*) Spacings of extracted wax (Å.)	(*b*) Spacings from examination of pupa (Å.)	Miller indices of plane
7·4	Lines lost in central blur	100
4·89		010
4·11	4·16	110
3·73	3·71	200
2·97	2·93	210
2·48	2·45	020
2·34	2·35	120
2·23	2·21	310
2·06	2·06	220
1·87	Lines too weak to be measured	400
1·75		320
1·61		130
1·51		230
1·49		500
1·43		510
1·37		330
1·27		520
1·23		140



Text-fig. 4. Plot of *a* and *b* unit cell dimensions against temperature, for wax obtained from chloroform washings of mature *Tenebrio* pupae.

Some results are shown in Table 2. It is clear that the material has a very wide range of melting-points and is probably a complex mixture. Examination of the tube in which the solution had evaporated showed that there had been a partial separation of different fractions of different melting-points. Around the top of the tube was a zone of hard white wax, which showed some change in appearance at 75° C. but did not melt until 81° C. This would seem to have been the material composing the surface wax filaments since these disappear at a similar temperature.

Table 2. *Changes in the appearance of samples of wax from chloroform washings of mature* Tenebrio *pupae, with increasing temperature*

(— = no change; × = slight changes; *t* = increased transparency; *m* = sample molten.)

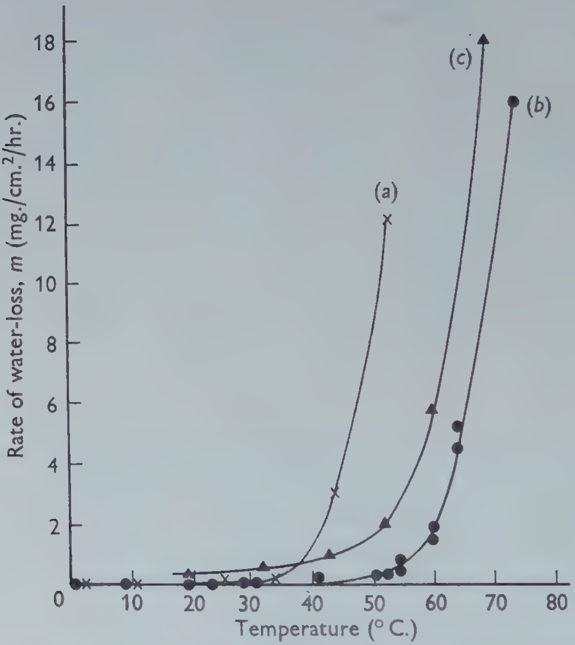
Temperature	Appearance of samples of wax								
	1	2	3	4	5	6	7	8	9
Room temp.	All samples solid, white crystalline								
Below 48° C.	—	—	—	—	—	—	—	—	—
48	—	—	—	—	×	—	—	×	×
53	—	—	—	—	*t*	—	×	*t*	*t*
55	—	—	—	×	*t*	—	*t*	*t*	*t*
59	—	—	—	*t*	*t*	—	*m*	*m*	*t*
62	—	—	—	*t*	*m*	×	*m*	*m*	*m*
65	—	—	—	*t*	*m*	*t*	*m*	*m*	*m*
68	—	×	×	*m*	*m*	*m*	*m*	*m*	*m*
75	×	*t*	*t*	.	.	.	.	.	.
80	*t*	*t*	*t*	.	.	.	.	.	.
82	*m*	*m*	*m*	.	.	.	.	.	.

Lower down there was a zone of material melting at 58–60° C., while mixed with it at the base of the tube were further crystals melting at 65° C. The range of melting points recorded in these optical studies thus agrees closely with that found from the diffraction experiments. In the optical studies appearance changes were frequently noted at temperatures well below the melting-points of the crystals (Table 2). In this Beament's (1945) observations were confirmed. However, the diffraction experiments on the same material, whilst yielding an identical range of melting-points, provide no evidence for a transition of the Müller (1932) type. There is certainly no such transition occurring for any major component of the wax at the temperature of the optical changes. The evidence thus appears to be incompatible with Beament's physico-chemical explanation of his observations, a fact which cannot but have important implications when the waterproofing functions of insect waxes are considered.

The surface wax of *Tenebrio* pupae is, therefore, chiefly composed of long chain hydrocarbon molecules, but these vary greatly in nature. Since insect waxes are mixtures containing not only paraffins but also a large proportion of alcohols and esters with some fatty acids, no chain length can be assigned to any of the fractions of different melting-points on the basis of properties of the pure paraffins. It is most unlikely that these mixtures will show sharp melting-points when in place on the insect.

THE WATERPROOFING PROPERTIES OF *TENEBRIO* WAX LAYERS

Measurements of the rates of water-loss in dry air, expressed in terms of weight loss per unit area of surface per unit time, have been made using the method of Wigglesworth (1945). The *Tenebrio* pupae studied were all killed by exposure for 30 min. to hydrogen sulphide gas, before beginning the experiment. It proved impossible to seal the spiracles effectively, since the thoracic openings lie deep among the delicate wing buds and appendages and cannot be reached without risk of damage to the cuticle.



Text-fig. 5. Graphs of rate of water-loss against temperature for: (*a*) newly moulted *Tenebrio* pupae, (*b*) mature *Tenebrio* pupae, (*c*) mature *Tenebrio* pupae washed in cold chloroform for 1 min.

Text-fig. 5 (*a*) shows the rate of water-loss from newly moulted (less than 1 hr. old) *Tenebrio* pupae, at various temperatures. The animal is efficiently waterproofed at low temperatures, but water-loss increases rapidly above about 35–40° C. and this would, following the work of Wigglesworth (1945) and Beament (1945), be held to indicate that the wax of the primary layer undergoes some change of phase at a 'critical temperature' of about 38° C. This value of 38° C. is reasonably close to the melting-point of the wax of the primary layer, as found by the diffraction studies.

If similar measurements are made using dead, mature *Tenebrio* pupae in which the surface wax secretion is complete, instead of young pupae, a different curve is obtained. Text-fig. 5(*b*) shows the plot of rate of water-loss against temperature for such insects. The rate of water-loss remains low (below 0·2 mg./cm.^{2}/hr.) up to a temperature of about 50° C., and then rises rapidly. Again, this would be held to indicate that the wax of the waterproofing layer undergoes a phase change at about 50° C., and this temperature would be referred to as a 'critical temperature'. We have already shown, however, that the hypothesis that this phase change is a crystallographic transition in the sense of Müller (1932) and Beament (1945) is not applicable to *Tenebrio* waxes. Furthermore, the waxes of the *Tenebrio* pupa are so complex in their spatial arrangement and their composition that it is unlikely that they will show any single melting-point. It is now necessary to consider what the true meaning of the 'critical temperature' is likely to be under these conditions.

In the first place, is the 'critical temperature' of a water-loss/temperature curve a genuine discontinuity? The curves published by Wigglesworth (1945), Lees (1947), and others, on which the concept of the 'transition temperature' is founded, certainly approximate very closely to exponentials. An exponential relationship between permeability and temperature is also to be expected on general theoretical grounds (Barrer, 1941). The general equation for diffusion of a fluid through a sheet of solid material is (Barrer, 1941, p. 5)

$$VC_g = \frac{D(C_1 - C_2)\,t}{L},$$

where C_g is the concentration of fluid which has diffused through the sheet into a volume V after a time t. C_1 and C_2 are the concentrations of fluid on either side of the sheet, D is the diffusion coefficient, and L the thickness of the sheet. For a gas this can be written in terms of pressures

$$\frac{Vp}{t} = \frac{D(p_1 - p_2)}{L}.$$

Assuming the gas is ideal, we can write

$$pV = mRT,$$

where m is the mass diffusing and R is the gas constant/g. The rate of water-loss, expressed as a mass/unit area/unit time, can therefore be written as

$$m = \frac{D(p_1 - p_2)}{LRT}.$$

Now p_1 is the vapour pressure of water on the inside of the cuticle, and is assumed to be equal to the saturation vapour pressure of water at the particular temperature T at which measurements are carried out. p_2 is put equal to zero, since the insect is in a desiccator. A check that diffusion of water away from the surface is not an important factor was carried out by vigorously stirring the air in the desiccator. This had no detectable effect on the rate of water-loss.

The vapour pressure of water depends approximately exponentially upon temperature

$$p = A\,e^{-B/T},$$

where A and B are constants ($B = 5200$), and we may, therefore, write

$$m = \frac{DA}{LRT} e^{-B/T}.$$

Furthermore, D may be temperature dependent. For many systems

$$D = D_0 \, e^{-E/T},$$

where D_0 and E are constants. If D does depend exponentially on temperature in this way, the temperature dependence of the rate of water-loss becomes

$$m = \frac{X}{T} \, e^{-Y/T},$$

where X and Y are constants. Now the major part of the temperature variation of m in this expression arises from the exponential term, whilst the T in the denominator outside the exponential has a relatively small effect on the shape of the curve. We may, therefore, neglect its variation and write

$$m = Z \, e^{-Y/T},$$

where Z is taken to be constant. Indeed, considering the uncertainties inherent in the tentative assumption that

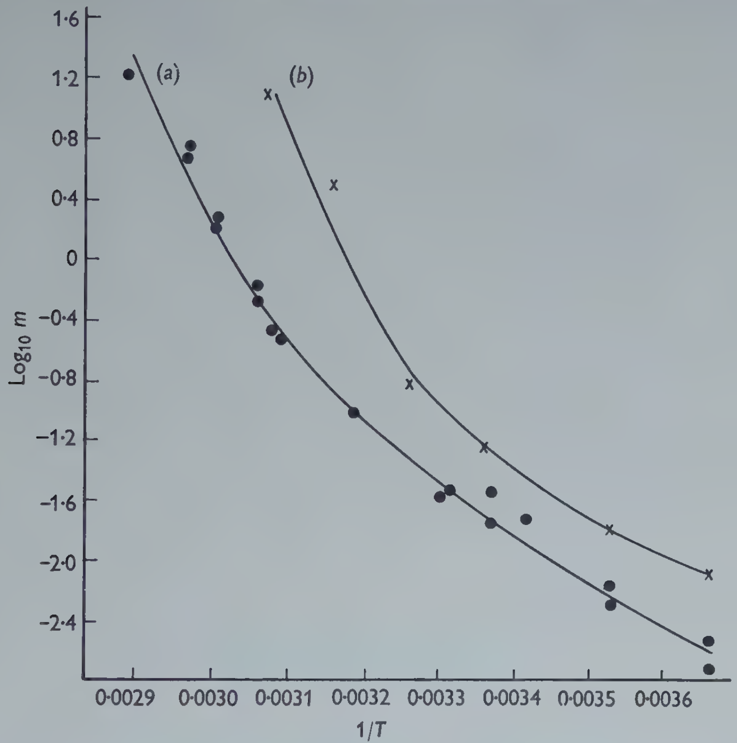
$$D = D_0 \, e^{-E/T},$$

it would be unwise to attach any significance to the $1/T$ appearing outside the exponential. Moreover, the very existence of this term depends on how D is defined.

In order to test the validity of the above theory we plot $\log_{10} m$ against $1/T$ and examine the graph for any departure from linearity. In plotting such a graph it is essential to use only results from pupae which have been reared under carefully controlled conditions. If results obtained from pupae taken from stock cultures are used, the scatter of points on the logarithmic plot is such that no useful conclusions can be drawn. However, if one uses data from groups of animals which had been transferred to clean glass tubes whilst in the larval stage prior to pupation, the experimental error is considerably less. Text-fig. 6 (*a*) shows the plot of $\log_{10} m$ against $1/T$ for groups of mature *Tenebrio* pupae reared in this way. A similar logarithmic plot of results from a single group of newly moulted *Tenebrio* pupae, is shown in Text-fig. 6 (*b*).

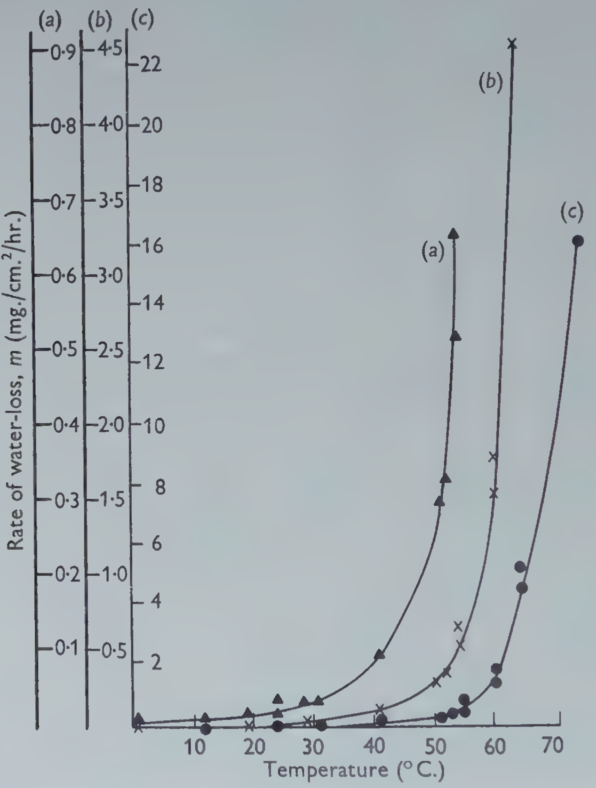
In both these curves the experimental points on the logarithmic plot depart significantly from linearity. However, before one can decide on the reality of the 'critical temperature' it is necessary to decide whether or not these logarithmic curves are smooth and continuous. Whilst it would be perfectly possible to fit two intersecting straight lines to the experimental points of either curve of Text-fig. 6 it is equally possible to fit these data with continuous curves. Since there is no *a priori* reason for postulating the existence of a 'critical temperature', one must assume that the curves are smooth and continuous as indeed they appear. We can, therefore, on the grounds of economy of hypothesis say that there is no evidence for a 'critical temperature' in the sense of a mathematical discontinuity in these insect water-loss curves. It is, however, possible and indeed likely that physical changes in the epicuticular waxes (e.g. partial melting) may produce changes in slope in the water-loss curves. Such changes would be likely to take place over a range of temperatures. They would thus give rise to a smooth curve rather than one showing a 'critical temperature', i.e. a definite temperature at which a transition of some kind occurs.

It is not safe to use the apparent sudden rise in a graph of water-loss against temperature (Text-fig. 5) as a criterion for a 'critical temperature'. The logarithmic plots show that log m increases more rapidly with increasing temperature than linearly with $-1/T$. This means that a direct plot of m against T is a graph which rises extremely rapidly with temperature. Such a graph when plotted on a particular (non-logarithmic) scale appears to rise very suddenly at some small range of abscissae, and in the case under consideration this had led to the concept of a 'critical temperature'. This sudden rise does not necessarily correspond to a discontinuity in the curve but merely means that the ordinates of the rapidly rising curve are becoming comparable in magnitude with the particular scale-units used for the m axis. Text-fig. 7 illustrates this point; the data of Text-fig. 5 (b) are plotted on three different scales, and the eye would judge different 'critical temperatures' in each case.



Text-fig. 6. Graphs of $\log_{10} m$ against $1/T$ for: (a) mature *Tenebrio* pupae, (b) newly moulted *Tenebrio* pupae.

The departure from linearity of the logarithmic plot might suggest that the physical theory outlined on pp. 95–96 is not fully applicable to insect cuticle temperature/permeability relationships. But, the departure from linearity is not very great, and the validity of the treatment is further attested by the findings of Holdgate (1955*b*) for aquatic insects. In these animals—the experimental material being drawn from a wide range of Orders and developmental stages—log m varies linearly with $1/T$ in all cases. Furthermore, linear log $m/(1/T)$ plots are obtained from *Tenebrio* pupae which have been lightly abraded with charcoal. It seems, therefore, that the theory gives a satisfactory general description of the permeability/temperature relationship of insect cuticles. It must be realized, however, that the assumption that



Text-fig. 7. Plot of water-loss data for mature *Tenebrio* pupae on three different scales, illustrating the effect of scale on the apparent position of the 'critical temperature'.

$$D = D_0\, e^{-E/T}$$

is not necessarily justified. There are many factors which could cause D to vary other than exponentially with temperature in a system so complex as the insect cuticle. In particular, it is known that insect waxes are complex mixtures, and that partial melting of these mixtures commences at comparatively low temperatures. Partial melting of the wax might be expected to produce an increase in the rate of water-loss. It would, however, be unwise to assign too much importance to the departure of the log $m/(1/T)$ plots from linearity, or to attempt a detailed explanation of the mechanism of insect water-loss, without further and more precise experimental evidence.

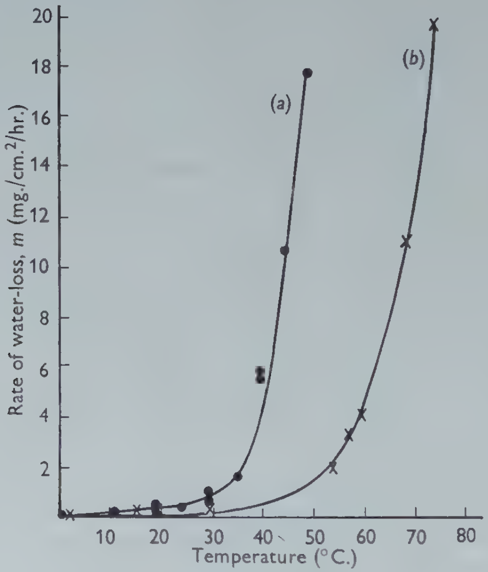
It is, however, possible to correlate the change in shape of the water-loss curve for *Tenebrio* pupae, illustrated by Text-fig. 5 (*a*) and (*b*), with the development of the outer epicuticle. It is found that this change occurs at about the 12 hr. stage and certainly happens before the secretion of the surface wax layer begins. Furthermore, mature pupae from which the surface wax layer has been removed by brief treatment with a cold solvent yield a curve which is perhaps more similar to that from the untreated mature pupae than to that from the young pupae (Text-fig. 5*c*). It seems, therefore, that the surface wax layer is not responsible for the additional waterproofing: the change in shape of the water-loss curves is in fact associated with the secretion of the cement layer rather than with that of the surface wax.

It is possible that the change in shape of the water-loss curves might be due to the evaporation of a volatile 'solvent' from the primary wax layer as occurs in samples of cockroach grease (Beament, 1955). However, if *Tenebrio* pupae are killed at moulting and then left exposed to the air for over 24 hr. the water-loss curve remains unchanged. Also, in several of the experiments on the water-loss/temperature relationship for the newly moulted pupae, the animals were heated to 50° C. and then cooled and the rate of water-loss at lower temperatures determined. The rates of water-loss are higher than those previously measured, but the curves do not resemble those for mature pupae. The wax of the primary layer crystallizes from solution in chloroform with a melting-point of about 40° C., and retains this low melting-point even in the vacuum conditions of the electron diffraction camera. It is known that heating or exposure to vacuum will accelerate solvent evaporation in *Periplaneta* (Beament, 1955).

If, therefore, the increased waterproofing of the mature pupa is due to a change in the composition of the primary wax layer, this change must involve further secretion of wax by the pupa: it cannot be due to a simple physical change in the primary wax itself. However, it seems far more likely that the increased waterproofing is due to the secretion of the layer of cement over the surface of the primary wax. If this is so, it is highly probable that the cement layer contains wax or some other hydrophobic material of low permeability to water. Indeed, Beament (1955) has shown the cement in *Periplaneta* to be composed of a shellac-like material. Shellac contains wax as a main constituent, and in *Laccifer lacca* itself the wax is even present in excess and forms a surface bloom. If *Tenebrio* pupal cement were similar to shellac, the transport of wax to form a new waterproofing layer and the appearance of the surface wax filaments would be equally readily explained. The latter would be due to the crystallization of excess waxes on the surface of the cement during its hardening. This hypothesis is attractive since it is otherwise necessary to postulate the secretion of the surface wax filaments through pre-formed primary wax and cement layers.

The waterproofing properties of other insect wax layers

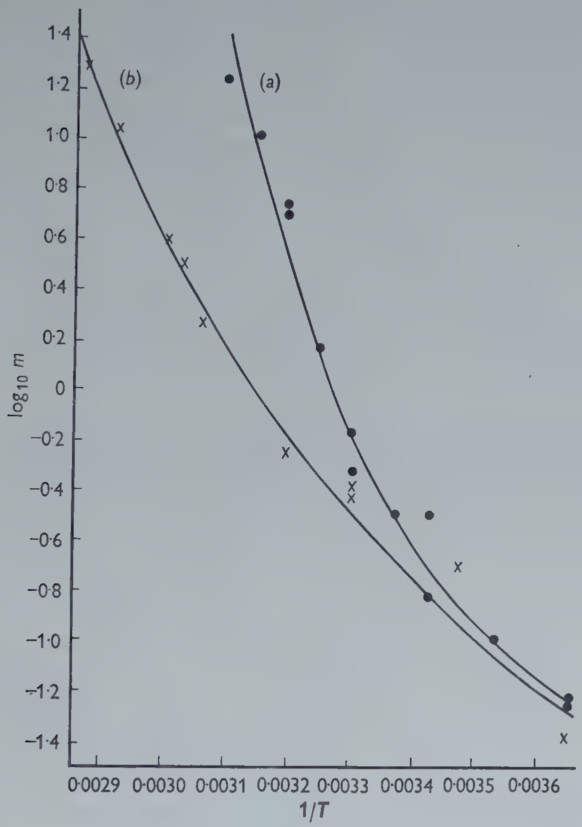
The primary wax layer of the *Tenebrio* pupa appears to be homologous with the layer which is exposed on the cuticle surface of the newly moulted *Rhodnius* (Wigglesworth, 1947), and in several other species. In these species, where there is not the complication of the surface wax layer, it has always been assumed that the primary wax layer is responsible for waterproofing the cuticle. The discovery of the large change in shape of the water-loss curves for *Tenebrio* pupae, occurring at the time of cement secretion, rendered some parallel work on other species desirable, in order to ascertain whether or not the change was peculiar to *Tenebrio*.



Text-fig. 8. Graphs of rate of water-loss against temperature for: (*a*) 30 min. *Rhodnius* nymphs, (*b*) 30 hr. *Rhodnius* nymphs.

A group of *Rhodnius prolixus* 5th-instar nymphs was killed by exposure to hydrogen sulphide gas within 30 min. of moulting. At this stage animals briefly washed in cold chloroform will blacken on immersion in ammoniacal silver nitrate, showing that an exposed wax layer is present. When the curve for water-loss against temperature is plotted (Text-fig. 8*a*) the rate of water-loss is found to rise to high values at even lower temperatures than in *Tenebrio* pupae at the same stage. On the other hand, a group of nymphs from the same batch which were fed at the same time and which moulted at the same time, when killed and examined 30 hr. after moulting, showed good waterproofing to comparatively high temperatures (Text-fig. 8*b*). A group killed and examined at about the 4 hr. stage yielded an intermediate curve. The data of Text-fig. 8 are plotted as $\log_{10} m$ against $1/T$ in Text-fig. 9, and give lines of slight curvature similar to those obtained from *Tenebrio* pupae.

These results establish conclusively that the change occurring in the water-loss curves for *Tenebrio* pupae at the time of cement secretion is in no way a unique feature of that animal. In *Rhodnius* there is a similar correlation between the time of the change and the secretion of the cement. Solvent evaporation as a cause of the change can again be ruled out, as animals killed soon after moulting and left exposed to the air for 24 hr. retain the water-loss curve of the very young nymph.



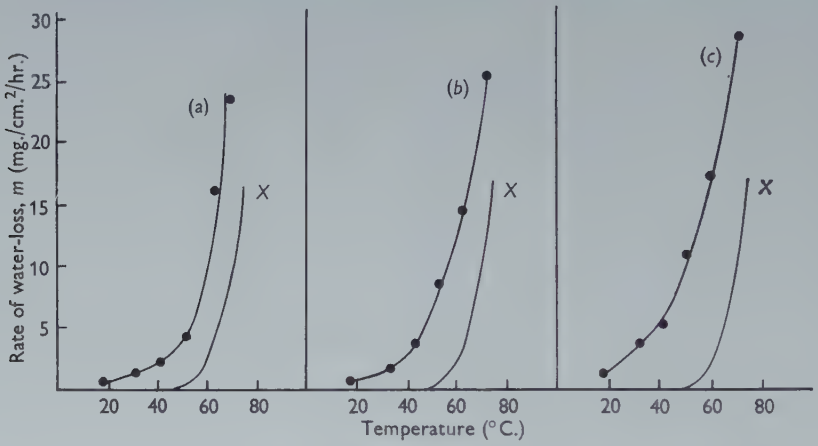
Text-fig. 9. Graphs of $\log_{10} m$ against $1/T$ for: (*a*) 30 min. *Rhodnius* nymphs, (*b*) 30 hr. *Rhodnius* nymphs.

The water-loss curves of lightly abraded Tenebrio *pupae*

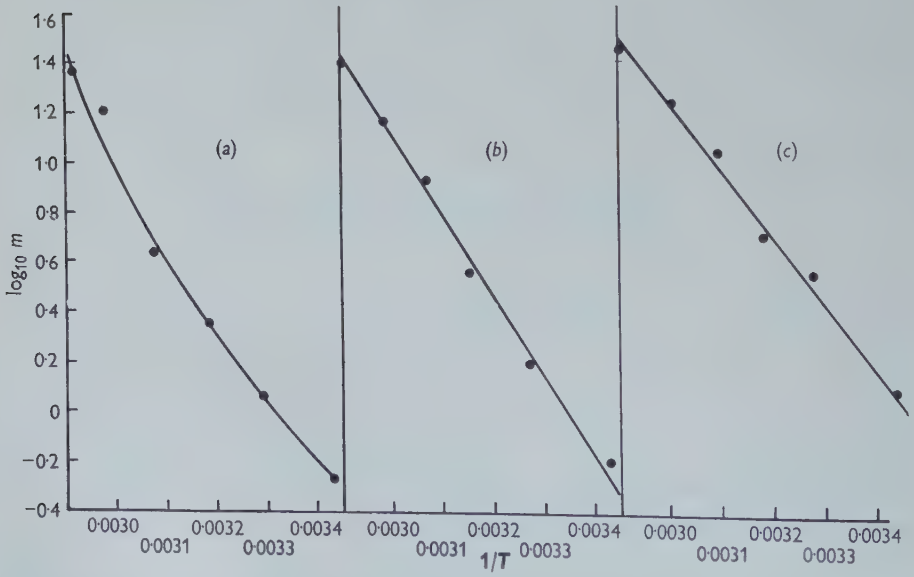
Abrasion by mineral dusts greatly increases the rate of water-loss from insects, and even quite gentle treatments may have a considerable effect (Wigglesworth, 1945). The effect of such treatment on the water-loss/temperature curve for *Tenebrio* pupae is of considerable interest.

Dusting was carried out by placing the dead pupae together with a little freshly ground activated charcoal in a clean, dry boiling tube. The tube was then corked, and the insects and dust were rolled from end to end of the tube a known number of times before being tipped out and transferred to gauze trays in which they were weighed and placed in a desiccator. By varying the number of times the pupae were rolled along the tube, a rough control of the intensity of dusting could be achieved.

Text-fig. 10 shows the water-loss/temperature curves for pupae rolled along a tube 10, 25, and 100 times, as compared with normal intact specimens. These data are plotted as $\log_{10} m$ against $1/T$ in Text-fig. 11. The treatments have increased the



Text-fig. 10. Graphs of rate of water-loss against temperature for mature *Tenebrio* pupae which have been abraded by rolling them along a tube containing charcoal dust: (*a*) rolled along tube 10 times, (*b*) rolled along tube 25 times, (*c*) rolled along tube 100 times. The lines marked X are the water-loss curves for intact, unabraded *Tenebrio* pupae.



Text-fig. 11. Graphs of $\log_{10} m$ against $1/T$ for mature *Tenebrio* pupae which have been abraded by rolling them along a tube containing charcoal dust: (*a*) rolled along tube 10 times, (*b*) rolled along tube 25 times, (*c*) rolled along tube 100 times.

permeability of the insects. It can also be seen that the more heavily dusted specimens show a linear relationship between log m and $1/T$. Furthermore, increased dusting causes log m to rise less steeply with $-1/T$, i.e. the coefficient Y in the equation

$$m = Z\,e^{-Y/T}$$

decreases with heavier dusting.

Values of Y for the curves of Text-figs. 10 and 11 are given in Table 3. As might be expected, increased dusting causes the curves to approach, in form, the saturation vapour pressure curve for water in which $Y = B = 5200$ (i.e. $E = Y - B = 0$, or D is not temperature dependent). There is thus a correlation between the permeability of the abraded cuticle and the temperature sensitivity of diffusion through it. The result is similar to that obtained from studies on aquatic insects (Holdgate, 1955*b*). A similar relationship also holds for the diffusion of many gases through rubber membranes (Barrer, 1941).

Table 3. *Values of E and Y for groups of mature* Tenebrio *pupae after abrasion by rolling along a tube containing powdered charcoal*

Number of times rolled along tube	Gradient of $\log_e m/(1/T)$ curve $= Y$	$E = Y - B = Y - 5200$
10	7600*	2400
25	7500	2300
100	6200	1000
Vapour-pressure curve	5200	—

* Mean gradient of slightly curved line.

DISCUSSION

The epicuticular layers of the *Tenebrio* pupa conform to the general morphological pattern recognized by Wigglesworth (1948*b*) and Dennell & Malek (1953). The surface wax layer, which is the chief peculiarity of the animal is present above a cement layer of normal type, below which there is a primary wax layer in the usual position. The actual form of the surface wax layer is peculiar, but granular surface waxes have been described from the Odonata by Becker (1941) and filaments of wax are commonly extruded during recovery from abrasion (Wigglesworth, 1945).

But, while all the normal layers of the outer epicuticle are present in the *Tenebrio* pupa, it appears that they are less distinct in structure and function than has previously been supposed. The primary wax layer alone does not seem to be responsible for the waterproofing of the mature cuticle; the cement may well play an important part in waterproofing besides acting as a protective sheet. If the cement does contain wax as a component, and if the wax of the surface layer does separate from it, it is likely that these two layers merge rather gradually into one another, and the cement may grade equally into the primary wax layer. It may be, therefore, that instead of having three distinct layers, we are dealing with a single 'outer epicuticle' of which the main component is wax, but which contains a central zone of bound carbohydrates (of shellac), giving strength and solvent resistance.

There is no doubt that the waterproofing of the insect cuticle is due to wax layers. The demonstration by Beament (1945) that layers of extracted waxes on membranes reproduce the water-loss/temperature curves of the insects from which they are derived established this. In the present paper it has been shown that disruption of the wax layer by dust abrasion alters the form of the water-loss curves for *Tenebrio* pupae and removes the non-linearity of the log $m/(1/T)$ plots. This might suggest that the great temperature sensitivity of diffusion which is associated with curvature of the logarithmic plot is a feature of an intact wax layer.

Finally, perhaps the most important outcome of the present study is that it has proved possible to relate the waterproofing properties of insect cuticles to those of other barriers to diffusion, and to indicate a general physical treatment which appears to give a valid description of many insect permeability phenomena.

SUMMARY

1. A study has been made of the structure of the outer epicuticle of the pupa of *Tenebrio molitor* (Coleoptera), particular attention being paid to a surface wax layer.

2. In this study two electron optical techniques, reflexion electron microscopy and electron diffraction, have been used.

3. At moulting, the *Tenebrio* pupa has a wax layer exposed on its surface. The nature of this wax has been investigated by electron diffraction. Most of the wax melts at about 40° C., and there is no indication of a 'transition' below this temperature.

4. A cement layer is secreted soon after moulting. Later, a surface wax layer, made up of numerous fine filaments, appears. These filaments melt at about 80° C., and are removed by wax solvents. Material obtained from chloroform washings of mature pupae shows a wide range of melting-points, but an investigation by electron diffraction provides no evidence for a 'transition'.

5. The validity of the concept of a 'critical temperature' in the water-loss curves is discussed. The experimental data can in every case be fitted by smooth and continuous curves. It is, therefore, suggested that any physical changes (e.g. melting) which may occur in the epicuticular waxes, take place over a range of temperatures rather than at a definite 'critical temperature'.

6. The water-loss curves for *Tenebrio* pupae change shape as the outer epicuticle develops. Mature pupae show good waterproofing to a higher temperature than do young pupae. This change in the curves is associated with the secretion of the cement layer.

7. Parallel studies have been carried out on *Rhodnius* nymphs and similar results obtained.

We would like to thank Prof. V. B. Wigglesworth, F.R.S., and Dr F. P. Bowden, F.R.S., for facilities to carry out this work and for their continued interest in it. We are also most grateful to many colleagues, in particular Dr J. W. L. Beament, Dr J. A. Chapman, and Dr J. W. Menter for helpful discussion. Our thanks are also due to Queen's College and the Agricultural Research Council for financial support for M. W. H., and to Messrs Tube Investments, Ltd. for a research grant to M. S.

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EXPLANATION OF PLATES

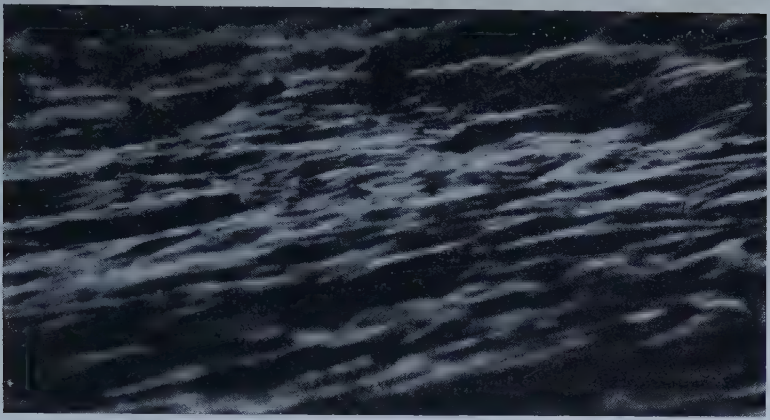
Pls. 1 (*a*), (*b*), (*c*) and 2 (*a*), (*b*) are reflexion electron micrographs of the epicuticular surface of *Tenebrio* pupae at different stages of development or after different treatments. The direction of illumination is from top to bottom of the surfaces as they appear in these micrographs. The magnifications $m_\parallel$ and $m_\perp$, therefore, refer to directions down and across the page respectively.

Plate 1

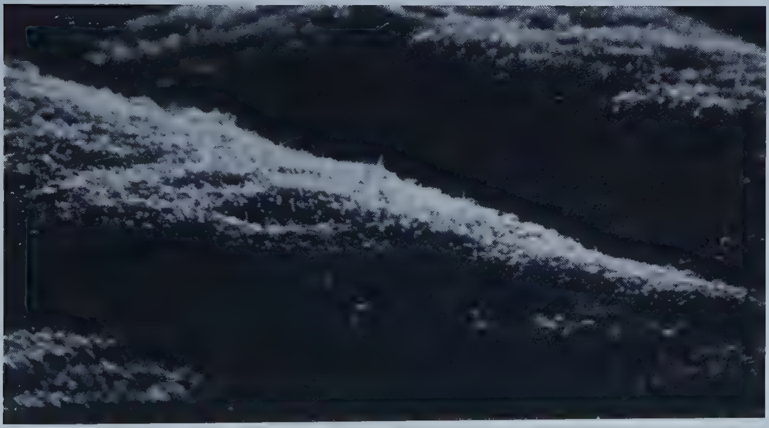
(*a*) Newly moulted pupa. $\beta = 11^\circ$, $m_\perp \sim \times 1500$, $m_\parallel \sim \times 250$.
(*b*) Mature pupa. $\beta = 11^\circ$, $m_\perp \sim \times 1300$, $m_\parallel \sim \times 200$.
(*c*) Mature pupa. $\beta = 5^\circ$, $m_\perp \sim \times 2500$, $m_\parallel \sim \times 170$.

Plate 2

(*a*) Mature pupa, after washing in cold chloroform. $\beta = 8^\circ$, $m_\perp \sim \times 1700$, $m_\parallel \sim \times 200$.
(*b*) Mature pupa, after heating to above 80° C. $\beta = 11^\circ$, $m_\perp \sim \times 2000$, $m_\parallel \sim \times 350$.
(*c*) Transmission electron diffraction pattern of wax obtained from cold chloroform washings of mature *Tenebrio* pupae.



(*a*)



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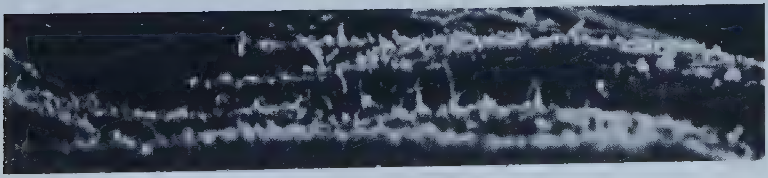
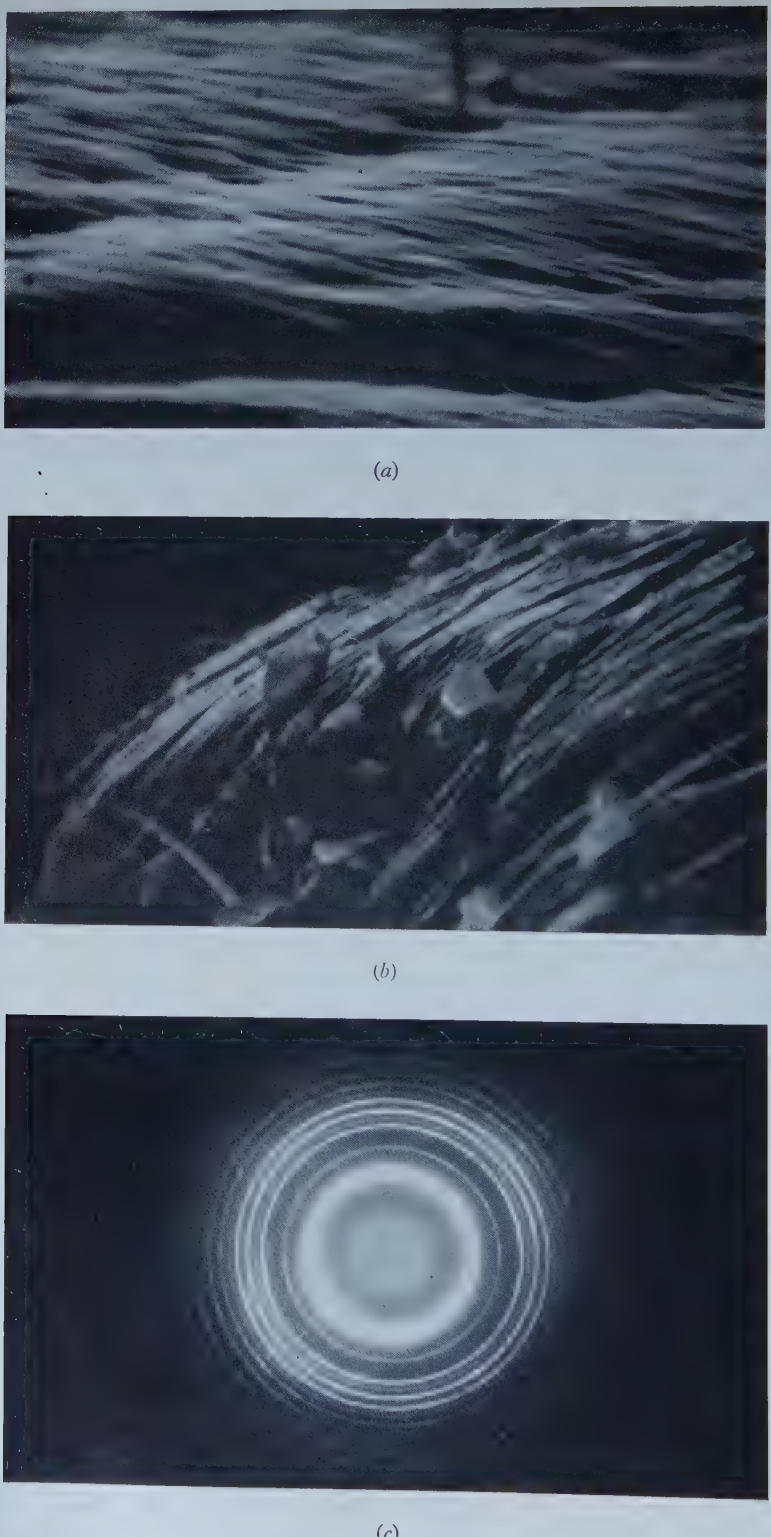


(*c*)

HOLDGATE AND SEAL—THE EPICUTICULAR WAX LAYERS OF THE PUPA OF *TENEBRIO MOLITOR* L.

(*Facing p.* 106)



(a)

(b)

(c)

HOLDGATE AND SEAL—THE EPICUTICULAR WAX LAYERS OF THE PUPA OF *TENEBRIO MOLITOR* L.

TRANSPIRATION THROUGH THE CUTICLES OF SOME AQUATIC INSECTS

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(*Received* 22 *August* 1955)

INTRODUCTION

The resistance of terrestrial insects to desiccation is well known to be due to a layer of wax in the epicuticle (Wigglesworth, 1945; Beament, 1945; Way, 1950; Wolfe, 1954; Holdgate & Seal, 1955). This wax can be extracted by solvents and will waterproof membranes on which it is deposited. Both intact insects and their extracted waxes on membranes show a similar, steeply rising, near-exponential relationship between permeability and temperature. Curves of the same type have been obtained from ticks (Lees, 1947), spiders (Edney & Davies, 1952), insect eggs (Beament, 1946, 1948), and mite eggs (Beament, 1951), and wax layers have been demonstrated in the majority of these groups.

But this combination of a low rate of water loss and a high temperature dependence of permeability is only known from undamaged terrestrial arthropods living in dry habitats. It does not occur in soil-inhabiting insects, owing to abrasion of the wax layer by the medium (Wigglesworth, 1945), or in abraded terrestrial insects (Holdgate & Seal, 1955), or in certain other terrestrial arthropods (Edney, 1951). Very little is known about cuticular permeability in aquatic insects, but what observations there are suggest a fairly high level of permeability (Harnisch, 1934; Wigglesworth, 1933). Recently, Shaw (1955) has demonstrated that the larva of *Sialis lutaria* is permeable to water both when exposed to dry air and when under the supposedly more natural conditions of a water/heavy water system. In this larva also, the exponential curve relating permeability and temperature rises less steeply than in terrestrial species.

It is important that further information should be obtained about the waterproofing mechanism in aquatic insect cuticles. If the group as a whole should prove to have permeable cuticles, then the correlation between waterproofing mechanism and habitat attains a greater significance. If they do not, it would suggest that a wax layer, once evolved, had been retained as a protection against the entry of water under osmotic forces. Further, since recent work has necessitated a reinterpretation of the 'critical temperature' (Holdgate & Seal, 1955), the study of as wide a range of insect cuticles as possible has become increasingly important.

WATER LOSS FROM AQUATIC INSECTS IN DRY AIR

Material and methods

The method of Wigglesworth (1945) has been used to determine the rates of water loss in dry air from a number of aquatic insects. Phosphorus pentoxide was used as the desiccating agent, and in later experiments the apparatus was modified by the introduction of a mechanism for stirring the air and setting up a circulation in the desiccator. The final arrangement is shown in Fig. 1.

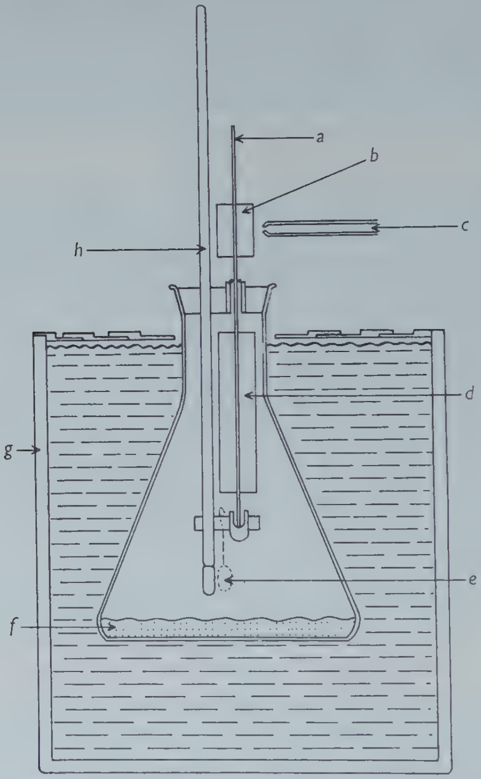

Fig. 1. Apparatus for measuring rates of water loss from insects. *a*, glass fibre; *b*, paper sail; *c*, air jet; *d*, paper sail stirring air in desiccator; *e*, specimen; *f*, phosphorus pentoxide; *g*, water bath; *h*, thermometer.

Rates of water loss over a wide range of temperatures were measured for the following species:

Anax imperator (Odonata; Anisoptera) nymph
Coenagrion puella (Odonata; Zygoptera) nymph
Limnophilus sp. (Trichoptera) larva
Corixa punctata (Hemiptera) adult
Corixa sp. (Hemiptera) adult
Notonecta obliqua (Hemiptera) adult
Agabus bipustulatus (Coleoptera; Dytiscidae) adult
Hydrobius fuscipes (Coleoptera; Hydrophilidae) adult
Sialis lutaria (Neuroptera) larva
Tipula sp. (Diptera) larva

All specimens were killed by exposure to hydrogen sulphide gas for about 1 hr., this agent being used because it combines high toxicity with an absence of components likely to affect the cuticle. The dead insects were suspended in the desiccator by loops of nylon thread tied loosely around the body; as a control measure to test that this did not damage the cuticle several other groups were suspended in gauze trays.

Rates of water loss at 20° C.

The rates of water loss from the species studied, expressed as mg./cm^2./hr. at 20° C. are given in Table 1. These figures are not precise, the chief causes of inaccuracy being the difficulty of measurement of surface area, and the fact that in some cases measurements were not made at exactly 20° C. so that the values have had to be obtained by extrapolation. But the errors certainly do not amount to more than 50% and are probably much less, so that the values quoted are sufficient for comparative purposes.

Table 1. *Approximate rates of water loss (mg./cm.^{2}/hr.) at 20° C. for the aquatic insects studied*

Species	Total no. of specimens	No. of experiments	Mean rate of water loss
Tipula sp.	5	2	11·5
Limnophilus sp.	2	1	11 approx.
Sialis lutaria	16	3	6·4
Coenagrion puella	30	4	4·2
Notonecta obliqua	19	6	2·9
Corixa punctata	23	4	2·1
Corixa sp.	6	1	1·8
Anax imperator	5	5	0·9
Agabus bipustulatus	9	2	0·9
Hydrobius fuscipes	16	2	0·3

It is clear from the table that a very wide interspecific variation in cuticle permeability exists in aquatic insects. But all the species are more permeable than normal terrestrial insects. The rate of water loss from *Tenebrio* pupae at 20° C., for example, is 0·02 mg./cm^2./hr., a figure which is less than one-tenth that of the least permeable aquatic insect and one five-hundredth that of the most permeable.

The rates of water loss from *Tipula*, *Limnophilus* and *Sialis* are nearly of the same order as those observed from a small capsule filled with water and exposed under the same conditions. But, as Shaw (1955) has pointed out, water loss from such a 'free-water surface' is only a small fraction of the theoretical figure, the limiting factor being the slow rate of diffusion of water vapour away from the surface. It is obviously important to ascertain whether gaseous diffusion is similarly limiting for the more permeable insects, and the introduction of the air-stirring mechanism in the desiccator allowed this to be done.

For a free-water surface, stirring the air increases the rate of evaporation considerably. In one experiment, water loss from a polythene capsule brimful with water rose from 120 to 180 mg./cm^2./hr. when the air was stirred, and reached

600 mg./cm^2./hr. when the capsule was placed directly under a compressed air jet, even though this air was not dry. On the other hand, an *Anax* nymph placed under the same air jet lost water less rapidly than it did in an unstirred desiccator. A group of *Sialis* nymphs lost water at a rate of 6·6 mg./cm^2./hr. in still dry air, and at 7·5 mg./cm^2./hr. when the air was vigorously stirred. With *Notonecta* adults the effect was even less, and the figures obtained using still and stirred air fell on the same curve.

The results seem to indicate that the rate of gaseous diffusion from the cuticle surface to the desiccating agent is not the main limiting factor for water loss from aquatic insects. Instead, the actual permeability of the cuticle appears to be limiting; although the cuticles in these species are more permeable than those of terrestrial insects they do therefore exercise some restriction on the passage of water through them.

The relationship between water loss and temperature

The rates of water loss in dry, still air for the above species were determined over a wide range of temperatures. The curves obtained are plotted in Figs. 2, 3 and 4.

These curves are closely similar to that obtained for *Sialis* larvae by Shaw (1955), and they confirm that this animal is in no way atypical among aquatic insects.

In all the species studied water loss increases considerably with temperature, but the curves obtained have a continuous exponential form and show no sharp breaks or 'critical temperatures'. They closely resemble the curve for water-vapour pressure against temperature. This resemblance is not merely fortuitous. The rate of water loss, expressed as mass/unit area/unit time can be shown (Holdgate & Seal, 1955) to be governed by the equation

$$m = \frac{D}{LRT}(p_1 - p_2),$$

Where m = the rate of water loss, D the diffusion coefficient, L = the thickness of the solid barrier, R the gas constant, T the temperature, p_1 the vapour pressure of water on the inside of the cuticle, and p_2 the vapour pressure of water on the outside.

It is assumed that there is saturation on the inside of the cuticle, so that p_1 is equal to the saturation vapour pressure of water at temperature T. Since the insect is in a desiccator, p_2 is zero. Now, since p_1 increases exponentially with increasing temperature, while p_2 remains constant, the expression $(p_1 - p_2)$, and in consequence the rate of water loss, will also increase in this way.

In the more permeable aquatic insects—*Tipula*, *Sialis* and *Coenagrion*, for example—the increase in water loss with temperature follows a water vapour-pressure curve closely. When log m is plotted against $1/T$ for these species (for the basis of this treatment see Holdgate & Seal (1955)), the curve obtained has practically the same slope as one relating p_1 to $1/T$ (Figs. 5, 6). This suggests that in these examples the diffusion coefficient, D, does not vary very much with temperature.

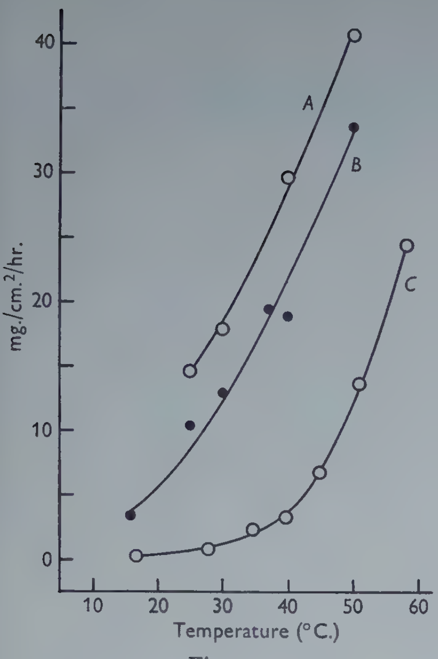

Fig. 2

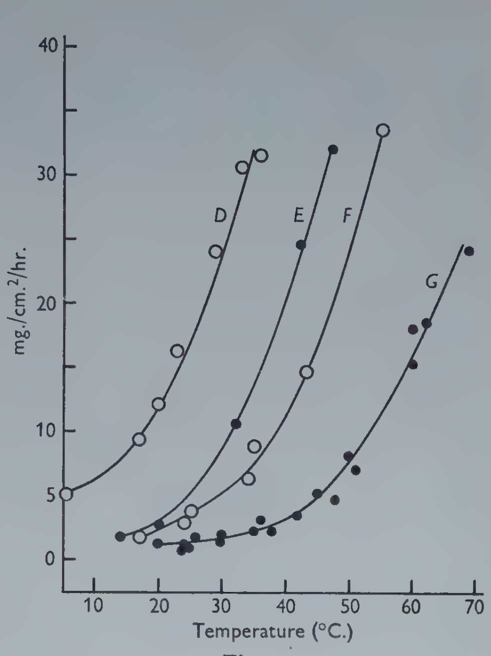

Fig. 3

Fig. 2. The variation in transpiration with temperature in three species of aquatic insect. *A, Limnophilus* sp.; *B, Coenagrion puella*; *C, Hydrobius fuscipes.*

Fig. 3. The variation in transpiration with temperature in: *D, Tipula* sp.; *E, Corixa punctata*; *F, Corixa* sp.; *G, Anax imperator.*

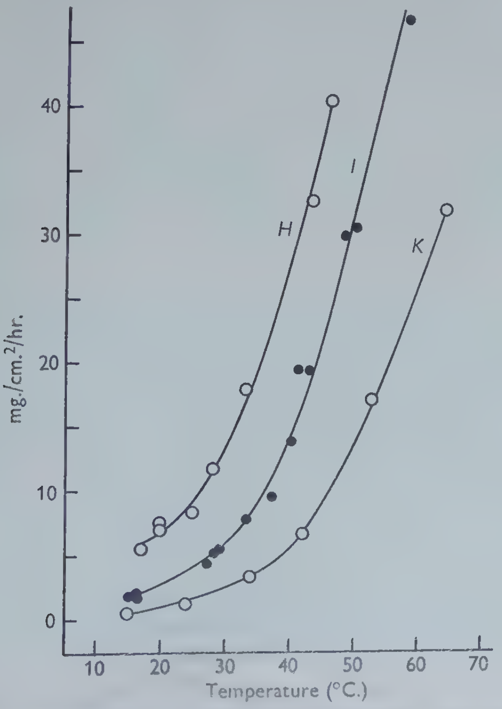

Fig. 4. The variation in transpiration with temperature in: *H, Sialis lutaria*; *I, Notonecta obliqua*; *K, Agabus bipustulatus.*

On the other hand, in the less permeable aquatic species, log m increases much more rapidly with increasing temperature, and the curves of log m/T^{-1} and log p_1/T^{-1} diverge (Figs. 5, 6). In these examples there appears to be an exponential temperature-dependence of diffusion, and the increasing water-vapour pressure difference cannot alone account for the increasing water loss.

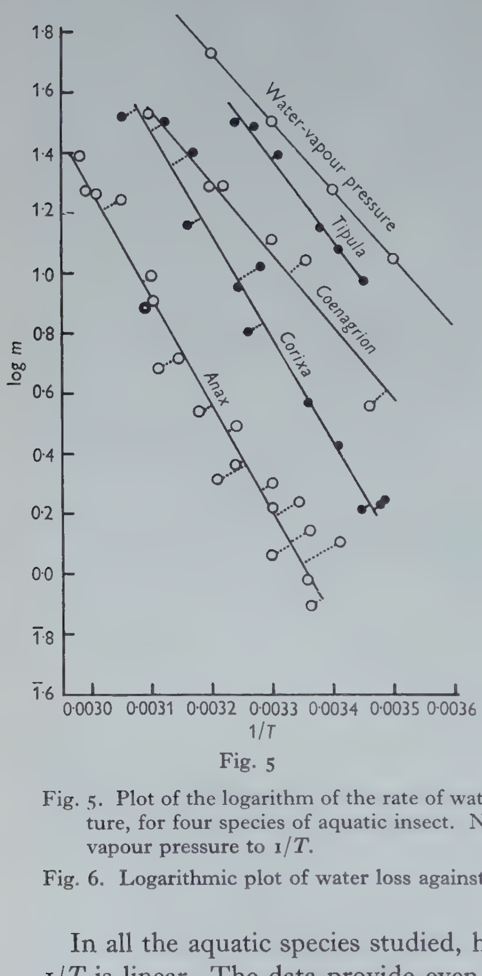

Fig. 5

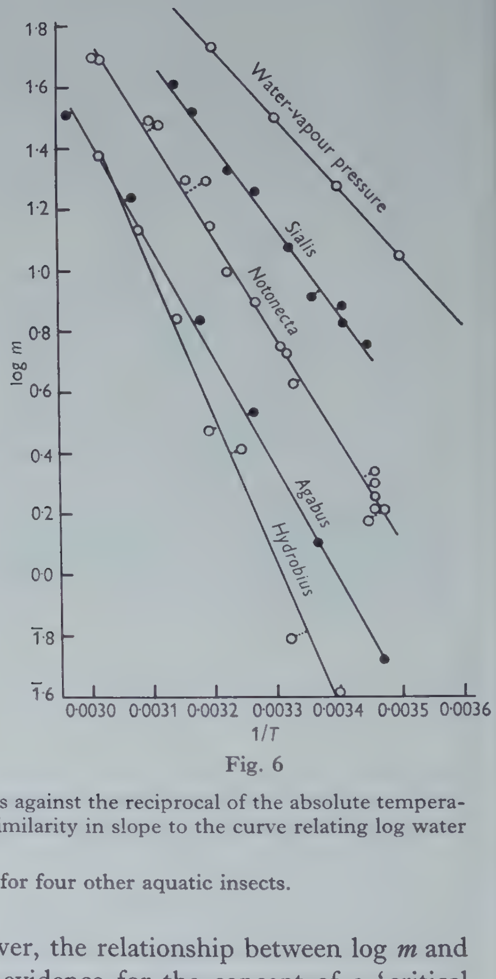

Fig. 6

Fig. 5. Plot of the logarithm of the rate of water loss against the reciprocal of the absolute temperature, for four species of aquatic insect. Note similarity in slope to the curve relating log water vapour pressure to $1/T$.

Fig. 6. Logarithmic plot of water loss against $1/T$ for four other aquatic insects.

In all the aquatic species studied, however, the relationship between log m and $1/T$ is linear. The data provide even less evidence for the concept of a 'critical temperature' than do those for water loss from terrestrial species. This linearity of the log m/T^{-1} relationship provides added evidence for the validity of the physical theory advanced by Holdgate & Seal (1955).

The insects studied show a correlation between the permeability of the cuticle and the temperature sensitivity of diffusion through it. In the most permeable species log m varies with $1/T$ only, in a way more or less parallel with vapour-pressure changes. In the least permeable species, *Hydrobius*, the curve for log m/T^{-1} is much steeper, while in the terrestrial species studied by Holdgate & Seal

(1955), which are the least permeable of all, the temperature-dependence of permeability reaches a maximum. This relationship is brought out in Fig. 7, in which the slope of the log *m* curves is plotted against the permeability of the cuticle at 20° C. A similar relationship holds for abraded terrestrial insects (Holdgate & Seal, 1955), and also for gas diffusion through rubber membranes (Barrer, 1941).

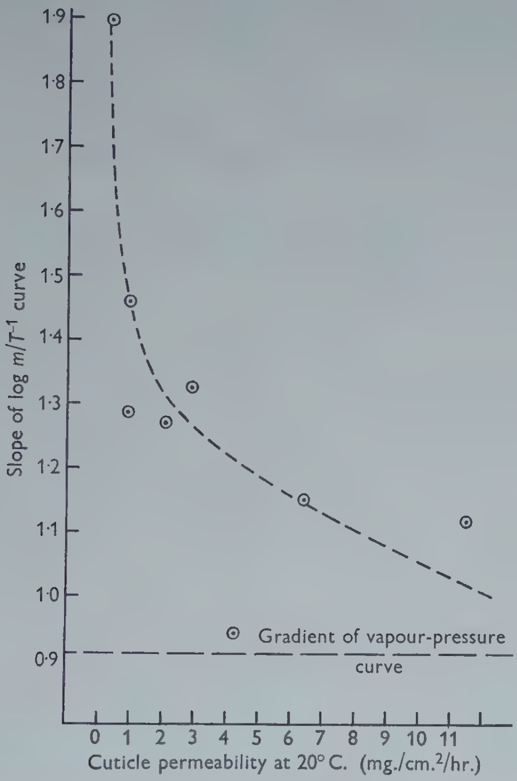

Fig. 7. The relationship between cuticle permeability and the temperature-dependence of diffusion (expressed in terms of the slope of the log m/T^{-1} curve). Trend line highly conjectural.

The effect of wax solvents on water loss from aquatic insects

In none of the data for aquatic insect water loss so far presented in this paper is there any evidence that wax layers are responsible for the resistance of the cuticles to the passage of water. Indeed, if the criteria for the presence of a wax layer were either very low permeability or the presence of a 'critical temperature', it could only be concluded that such a layer was absent. But neither of these criteria are in fact valid (Beament, 1954; Holdgate & Seal, 1955), and Shaw has produced some evidence for the existence of a lipid in the cuticle of *Sialis*, which he suggests may have a waterproofing function.

Table 2 shows the effect of brief immersion in chloroform at room temperature on the subsequent rates of water loss from a number of aquatic species. In all cases the treatment produced a considerable increase in transpiration, which in several instances rose to more than 5 times its previous level. This increase is of the same order as that produced by wax solvents in the cold on terrestrial insects, and might be held to indicate that the action of the solvent in the two cases was similar. If this is so, then exposed, chloroform-soluble material must play an appreciable part in the resistance of aquatic insect cuticles to the passage of water. If it is not so, then it must be admitted that chloroform can affect the permeability of cuticles in other ways than by solvent action.

Table 2. *The effect of chloroform on the evaporation of water from some aquatic insects. Animals immersed in the solvent for 2–5 min. at room temperature. Rates as* mg./cm.^{2}/hr.

Species	No. of specimens	Temperature (° C.)	Initial rate	After treatment
Notonecta obliqua	3	16	2·0	11·3
	3	16	2·2	11·8
	4	15	1·6	10·9
Corixa punctata	2	15	1·7	12·0
	4	14	1·8	9·4
Tipula sp.	2	22	14·0	17·0
Anax imperator	2	20	1·0	17·0
	2	20	—	16·5
	1	20	—	16·4
	1	20	1·1	11·6
Coenagrion puella	10	17	2·9	5·7

The effect of mineral dusts on water loss

In most terrestrial insects water loss through the cuticle is greatly increased by rubbing the animal with a mineral dust (Wigglesworth, 1945). This abrasion need not be very vigorous nor need the dust involved be very hard, since the impermeable wax layers lie close to the surface and are readily penetrated.

Table 3. *The effect of charcoal dust on water loss from some aquatic insects*

Species	No.	Treatment	Initial rate	Subsequent rate
Coenagrion puella	10	Light dusting	2·87	2·91
Notonecta obliqua	2	Abraded	2·0	3·7
	4	Abraded	2·5	3·0
Corixa punctata	5	Abraded	1·9	4·2

Table 3 shows the effect of abrasion with activated charcoal dust on certain aquatic insects. The dust was ground in a pestle and mortar and spread on filter-paper, and the insects were held lightly between finger and thumb and gently rubbed across the dusted paper for a standard distance. This treatment is sufficient to produce a 40-fold increase in water loss from *Tenebrio* pupae, when these are abraded in the same way, over about the same area of cuticle. But the table shows that in aquatic insects the increase following dusting is very much less—in some of the experiments so much less as to be of doubtful significance. It might therefore be concluded that the waterproofing layer, such as it is, is not accessible to the dust, although such a hypothesis does not agree with the results of the chloroform treatment. But the actual increase in water loss after abrasion is about the same in aquatic and terrestrial insects. It may be that equal areas of cuticle are abraded to about the same depth, but that the permeability of the aquatic insect is so great already that the proportionate increase is negligible.

Water uptake by an aquatic insect in distilled water

The measurement of cuticle permeability for aquatic insects in terms of weight loss in dry air is unrealistic in that it exposes the animals to conditions which they do not meet in nature. It was, however, necessary to use this method for comparison with terrestrial examples. In addition, as a check on the results, another method of estimating cuticle permeability was devised and used on adult specimens of *Notonecta obliqua*, since these were readily obtained and of convenient size.

The animals were killed by H_2S, and nylon threads tied round them loosely for ease of handling. They were then immersed in a beaker filled with distilled water, being kept below the surface by a glass plate. The beaker was kept at room temperature (*c.* 20° C.), and the animals generally remained in it for 24 hr.

When the animals were removed, the problem was to distinguish between the increased weight due to the actual absorption of water through the cuticle and that due to the adherence of a film over much of their surfaces. This was done by first blotting the animals on dry filter-paper. They were then weighed and transferred to a desiccator, also at room temperature, and thereafter regular weighings were carried out, at half-hourly intervals. During the early period of desiccation the animals lost weight rapidly; this was considered to be due to the removal of superficial moisture. But, after about 30 min. a steady rate of weight loss was attained, comparable with that shown by normal, dead insects, and this was held to be due to transpiration through the cuticle. By extrapolation of this steady rate back to the time of removal from the water, a figure for the actual amount of water taken up by the animal could be obtained.

RESULTS

Table 4 demonstrates that *Notonecta* shows considerable permeability to water under these conditions. In evaluating the results it must be remembered that much of the cuticle surface is covered with a hair pile, which normally retains air, and is only slowly invaded by distilled water. The area of cuticle involved in water uptake may therefore be only about half that concerned in water loss. The physical forces involved in the movement of water are also very different in the two cases. But, while these difficulties make a detailed comparison impossible, it is clear that the permeability of *Notonecta* cuticle is of the same order of magnitude in the two directions, and under the two sets of conditions.

In the desiccation experiments, where the spiracles were not sealed, there was probably an appreciable tracheal component of water loss. But the high rates of transpiration cannot be solely due to the tracheal system, for the cuticle is also shown to be permeable in the water uptake experiments, in which the tracheae remained filled with air.

Table 4 also demonstrates the effect of brief chloroform treatment on the subsequent rates of water uptake. In all cases this treatment produces an appreciable increase in permeability, although this is not so great as is observed under conditions of desiccation.

Table 4. *Water uptake by* Notonecta obliqua. *Exps.* 1–3, *untreated specimens. Exps.* 4–7, *specimens washed in chloroform at room temperature for* 2 *min.*

Exp.	No. of specimens	Rate of water uptake (mg./specimen/hr.)	Treatment
1	4	0·65	Untreated specimens
2	4	0·36	
3	5	0·35	
		0·54	
		0·61	
		0·39	
		0·39	
1–3	13	0·48	
4	4	0·78	Specimens immersed chloroform
5	4	1·30	
6	3	0·90	
7	3	1·77	
4–7	14	1·17	

DISCUSSION

There is no doubt that aquatic insects, of whatever species, group, or developmental stage, are permeable compared with terrestrial species. But there is, none the less, a wide range of permeability within the group, and the least permeable examples do not differ very markedly from the more permeable terrestrial species. This in itself suggests that aquatic and terrestrial species may form a continuous series, rather than two discrete groups. Such a view is supported by a study of the water loss/temperature curves. In all cases these have an exponential form, as is to be expected on theoretical grounds (Barrer, 1941; Holdgate & Seal, 1955). Further, the steepness of the exponential is correlated with the level of permeability of the cuticle; a relationship which is well known from non-biological diffusion systems (Barrer, 1941), and the terrestrial insects simply represent one end of the series, in which permeability is minimal, and its temperature-dependence maximal.

Porous membranes in general combine high permeability with a slight temperature-dependence of diffusion (Barrer, 1941). It might therefore be considered that the permeable aquatic insects have porous cuticles, and that the series linking these species with the terrestrial forms is one of decreasing pore size and pore density. If this is so, it would be expected that an artificial increase in the porosity of terrestrial insect cuticles would produce an increase in permeability and a reduction in temperature-dependence. This is in fact the case. Abrasion of terrestrial insects by mineral dusts can convert the steep exponential water loss/temperature curve that they normally show into a 'vapour-pressure' type curve comparable with that of permeable aquatic species (Holdgate & Seal, 1955). Yet these abraded insects are far from freely permeable, and their rates of water loss are enormously increased by treatment with cold chloroform, which presumably acts by removing the wax layer from some of the undamaged regions of the cuticle. In all these features, therefore, they resemble aquatic species.

As a tentative hypothesis, therefore, it may be suggested that the permeable aquatic insects have cuticles which contain a wax layer, but that this layer is porous. The pores provide an easier pathway for water loss than the wax layer, and as a result the steep and non-linear $\log m/T^{-1}$ curve which characterizes diffusion through the wax layer is masked. The less permeable species may perhaps have narrower pinholes through which diffusion is more temperature-dependent, while the terrestrial species may have a pinhole system which is still more reduced. But there are other possible systems which could equally well give a graded series of permeability and temperature-dependence of diffusion, and until a great deal more is known about the properties of complex polar insect waxes it would be unwise to lay any stress on this or any tentative explanation.

SUMMARY

1. Measurement of the rate of water loss in dry air from ten species of aquatic insects, representing six orders, has shown that the cuticles are in all cases more permeable than are those of typical terrestrial species. There is, however, a wide range of permeability within the group.

2. The rate of water loss increases exponentially with temperature. There is no 'critical temperature'. The steepness of the exponential relating transpiration and temperature is greatest in the least permeable examples.

3. Chloroform treatment produces a marked increase in water loss, but abrasion by mineral dusts has very little effect.

4. Adult specimens of *Notonecta obliqua* immersed in distilled water gain weight at a rate which suggests that the cuticle is comparatively permeable under these conditions also. The rate of water uptake is increased by chloroform treatment.

5. The results support the general theory advanced by Holdgate and Seal (1955), but no sound interpretation of the structural basis of cuticular permeability can yet be advanced.

I would like to thank Professor V. B. Wigglesworth, F.R.S., for his great interest in this work, and for many valuable suggestions. Mr M. Seal has provided most helpful guidance in the field of physical chemistry, and Dr J. W. L. Beament has assisted in many ways. Most of the experimental work was carried out in the Laboratory of the Freshwater Biological Association at Windermere, and I am most grateful to the Director, Mr H. C. Gilson, for giving me facilities to work there.

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ON THE LIGHT RESPONSE OF THE CHROMATOPHORE OF THE SEA-URCHIN, *DIADEMA SETOSUM* (LESKE)

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(*Received* 19 *April* 1955)

The responses of chromatophores to light stimuli have been reported in various kinds of animals. In almost all cases, the stimuli have been regarded as not affecting the chromatophore directly, but through nervous or hormonal mediation. Little is understood about chromatophore behaviour as a primary response to light.

In *Diadema*, as Millott has already reported (1950, 1952), the chromatophores distributed over the interambulacral zones expand or contract following a suitable period of light or dark treatment. According to Millott, a local expansion can be elicited following illumination by a light spot. This seems to indicate a direct response of the chromatophore to light. In Millott's experiment, however, since relatively large areas were illuminated (0·5–1·5 mm. in diameter), there remains the possibility that peripheral nerve endings may have been involved in the photic response. The present experiment is made in an attempt to investigate whether or not the chromatophore reaction may be really direct (i.e. intracellular).

MATERIALS AND METHODS

Sea-urchin species *D. setosum*, available at Misaki, seems to comprise two forms. Urchins used in these experiments were limited to the form which had 'globular-shaped' blue spots along the border between the ambulacral and the interambulacral zone and five 'white' patterns at the equatorial plane of the test in the interambulacral zones.* An area convenient for observation was between the genital pore and the white pattern. Since colour changes can be induced by dark-treatment independently of the central nervous system, as Millott reported, this area (about 0·5 cm. wide and 1·5 cm. long) was cut off from the test. It was then placed in a glass vessel and observed under the microscope.

For stimulation, light from a microscope lamp was thrown on the material by the following arrangement. The light, after passing through a water filter (2·5 cm. in thickness), was made to converge to 3μ in diameter by an optical system consisting of a diaphragm, a prism and four lenses. These components were arranged in the following order.†

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* The other form has 'rod-shaped' blue spots and 'red' patterns at the corresponding places; the chromatophore distributions in the first group used in the experiment are sparser and the reactions to the light clearer than the second form.

† Author's thanks are due to Mr T. Nakatsubo of the Olympus Optical Company, without whose co-operation this apparatus could not have been prepared.

(1) An aperture (0·3 mm. in diameter) in the diaphragm served as the image, 150–200 Lux in intensity.

(2) A high-power lens ($f=2$ mm.) reduced the size of diaphragm image.

(3) Two low-power lenses of long focal distance picked up the small image and formed its virtual image at a conjugate focus of an objective lens (5).

(4) Along the course of (3), a prism was inserted to bend the ray at right angles so as to make the beam enter the optical axis of the microscope.

(5) An objective lens (× 10), which was being used for observation, picked up the virtual image formed by (3) and focused its real image of 3μ in diameter at the plane of the focus of the microscope.

If chromatophores are generally illuminated the complete dispersion of the pigment from the concentrated state takes 20–30 min.; when kept in darkness its complete concentration from the dispersed state requires 1–1½ hr. Therefore, when a light spot was thrown on a part of a chromatophore with fully concentrated pigment (whether it was applied on the pigment-containing or the pigment-free part of the cell) the illumination was continued for 20–30 min. When the light spot was thrown on a part of a chromatophore when its pigment was dispersed, the illumination was continued for 1–1½ hr.

RESULTS AND DISCUSSION

Description of the reaction will be given with reference to Fig. 1 (A–F and G–I).

(A) The initial condition of the experiment. The whole field has been generally illuminated for some time and the pigment is largely dispersed. The spot of light is now placed on the middle part of the branch of no. 4 chromatophore in which the pigment is incompletely dispersed (marked with a cross) and the general illumination is cut off.

(B) The whole preparation has been in darkness for 1½ hr. except for the spot of light on no. 4 chromatophore (now marked with a circle). It is significant that the pigment remains dispersed in the illuminated area of the chromatophore. In one branch of no. 5 chromatophore the pigment remains dispersed, and since this occurs nearest the light spot, it is probably due to light scattered from the spot.

The light spot is then moved to a new position indicated with a cross.

(C) One hour after B. In chromatophores nos. 1, 2 and 6, the pigment disperses as far as the light spot (circle), while in the branches of chromatophores nos. 4 and 5 the pigment has become fully concentrated.

The light spot is again moved to a place shown by a cross at the end of C.

(D) One hour after C. The pigment in nos. 5 and 6 chromatophores is apparently contiguous at the point of illumination.

The light is then moved slightly toward the centre (cross-mark) of no. 6 chromatophore.

(E) One hour later. After the shift of light, granules of no. 6 chromatophore remain on the stimulated point, while in no. 5 cell it has become concentrated again; as in C.

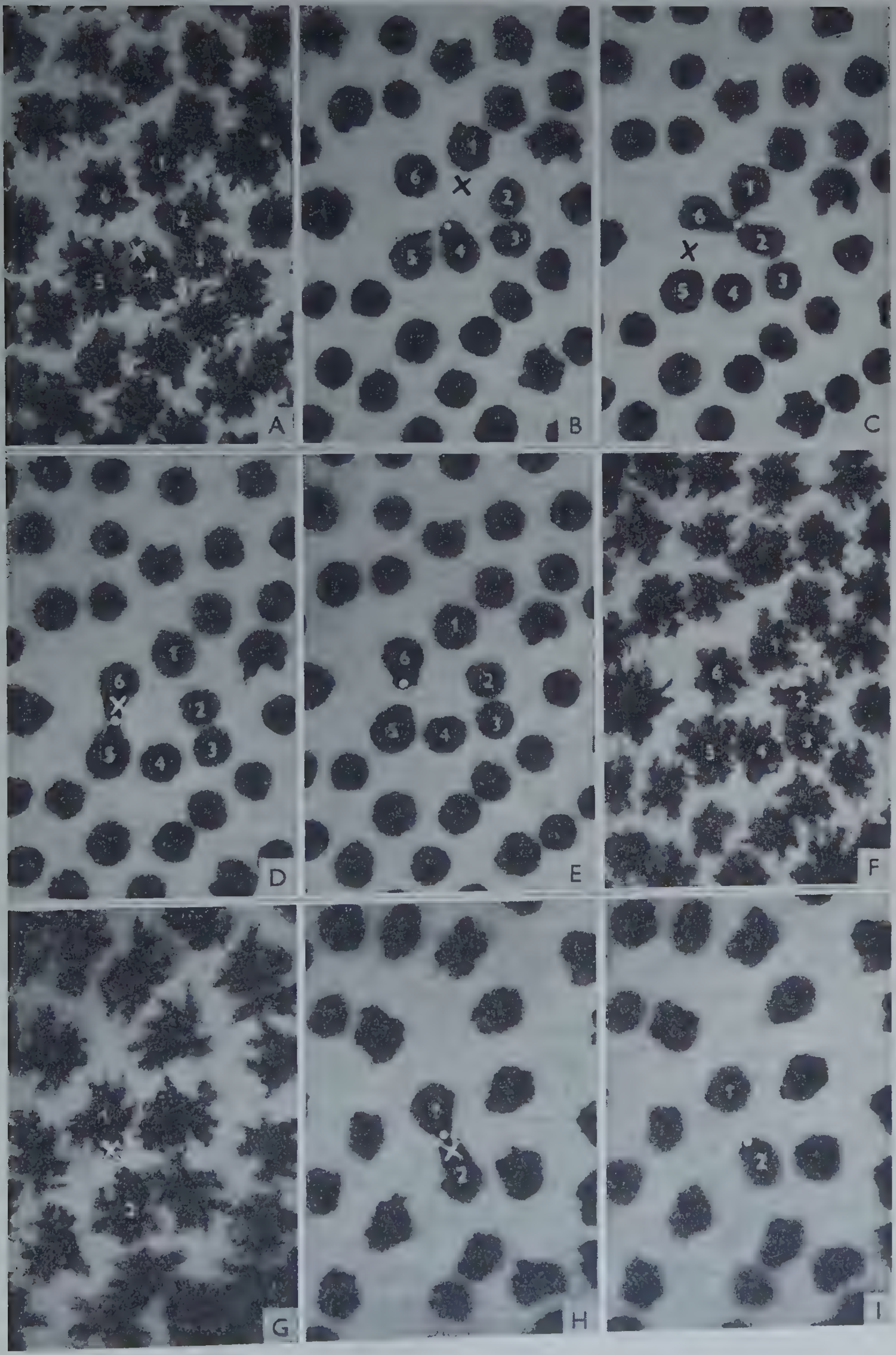

Fig. 1. Two series of experiments (A–F and G–I) in which spots of light are projected on various parts of chromatophores; temperature 16–18° C.; for explanation, see the text.

(F) Forty minutes after E—20 min. in darkness and 20 min. under illumination. The same condition as in A is resumed, with no sign of injury or fatigue. This is taken as evidence that the cells have been maintained in healthy condition, although the experiments from A to E lasted for 5 hr.

The next series:

(G) The initial condition after general illumination. The spot of light is then thrown on the distal end of the branch of no. 1 chromatophore in which the pigment is partly dispersed (marked with a cross).

(H) One and a half hours after G. Branches of nos. 1 and 2 chromatophores are apparently in contact at the stimulated point.

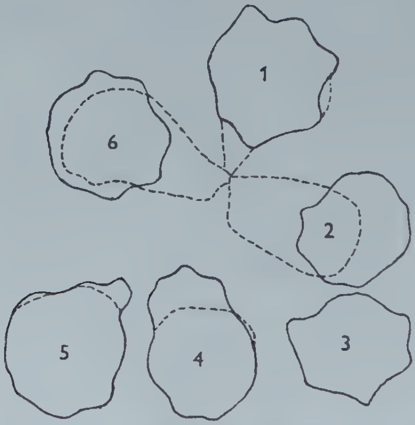

Fig. 2. Superposition of the outline of chromatophores of B (solid lines) and C (broken lines); for explanation, see the text.

The light spot is now shifted to a place shown by a cross.

(I) One hour after H. The light is at the margin of no. 2 chromatophore. In no. 1 cell the pigment is fully concentrated.

Another series of experiments shows that if the centre of the chromatophore is illuminated by the spot of light, the pigment does not disperse, but remains fully concentrated, regardless of its initial state.

The chromatophore behaviour described above can be summarized as follows: (1) when part of a chromatophore is illuminated, the pigment in that part cannot be withdrawn from the illuminated area: (2) when the pigment-free area of a chromatophore is illuminated, the cell disperses its pigment so as to cover the illuminated area. It may be inferred from these facts that the photic response of the chromatophore is direct; and further, that this photosensitivity is a property of the hyaloplasm since the presence of the pigment granules is not necessarily required in the illuminated part. Thus, the pigment granules have a capacity to move through the cellular fluid medium toward the illuminated point of the cell.

This interpretation may be more clearly illustrated if the above photographs of B and C are superimposed (Fig. 2). The darkward boundary of no. 2 chromatophore, which is reacting to the light spot placed far from its resting state, moves toward the light. Such is also the case for no. 6 chromatophore. These phenomena are observable in many other instances. The boundaries of nos. 2 and 6 chromatophores under any conditions, however, are indicated by areas of the pigment when fully dispersed. The shift of the chromatophore, described above, is apparent rather than real, and is due to a migration of pigment toward the lighted region of the cell.

It may be concluded that the chromatophores in *D. setosum* are photosensitive cells. However, it remains to learn whether the granules may themselves move toward the light or whether some other factor within the cell may initiate cytoplasmic movements which carry the granules.

SUMMARY

1. The nature of the photic response of chromatophores distributed over the test of *Diadema setosum* was investigated.

2. By applying a minute light spot to the various parts of single chromatophores, it was observed that the chromatophore can react locally to the light stimulus.

3. It is concluded that the photosensitivity resides within the chromatophore itself.

I wish to express my thanks to Prof. K. Dan, Prof. D. L. Fox, and Prof. N. Millott for their kindness in reading the manuscript.

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ENDOTHERMY AND ECTOTHERMY IN MICE AT −3° C.

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A study is being made of the effects on laboratory mice (*Mus musculus* L.) of breeding at an environmental temperature of −2 to −4° C. It has been found that both A (white) and C57 (black) mice, rigorously inbred, can be maintained indefinitely at this temperature, given cotton-wool for nesting (Barnett & Manly, 1954).

In general, adjustment to a low temperature may be of two kinds: (*a*) internal changes (endothermic), such as raised heat production or improved thermal insulation; (*b*) alteration of the environment of the animal (ectothermic), for instance nest-building or moving to a warmer place. This paper describes some observations on the combination of endothermic and ectothermic adjustments by which mice maintain themselves at −3° C.

MICE AND METHODS

The mice referred to in this paper were of the A strain, from two sources: the Imperial Cancer Research Fund (Mill Hill, London); and Glaxo Laboratories (Greenford, Middlesex). In one instance reference is made to C57 mice from Mill Hill. All breeding pairs were litter-mates, and were kept together, without other mice except their young up to 3 weeks old, from 5 weeks until they died or were killed. The two strains undoubtedly differ genetically (information on this point will be published separately); but for the purposes of this study they are treated together (with one exception mentioned later), since the differences between them did not influence the results described here.

The cold room was maintained at approximately −3° C., with a differential of 2° C; the temperature occasionally rose above 0° C., when work was going on. Control mice were kept in a warm room at approximately 21° C., with a differential of about 5° C. The relative humidity in both rooms was between 60 and 90%. Artificial light was on for 12 hr. each day; for the other 12 hr. the mice were in darkness.

The cages were rectangular metal boxes, about 36 × 15 × 11 cm. high, with wire lids carrying a water bottle and food basket. The floor was covered with sawdust, since the humidity was too high for peat moss. Except where mentioned, cotton-wool was supplied for nesting. The food was diet 41 (Bruce & Parkes, 1949). Water was supplied in bottles in the warm room, and in open jars, placed on the floor of the cage, in the cold room. When the water froze, the mice licked the ice.

Cages were cleaned twice a week, except that litters of less than 11 days were left undisturbed.

Body and nest temperatures were taken with copper-constantan thermocouples, with a reference couple at 0° C. For nest temperatures, the couple was enclosed in thin glass, to protect it from being gnawed by the mice. It was normally placed among the nestlings, as indicated for the highest temperatures shown in Figs. 3 and 4. Internal body temperatures were taken in the colon; for adults, the couple was always inserted to a depth of 3 cm.; for young, as far as it would go without causing injury (generally, 1·0–2·5 cm.). Insertion of a couple is helped if the mouse first discharges the formed faeces from the anus: this is especially important with males, since their faeces are harder than those of females. Skin and surface temperatures were taken with a thread thermocouple. For skin temperatures, the couple was placed on the skin surface in the middle of the back, and the hair flattened over it before the reading was recorded. For surface temperatures the couple was placed lightly on the hair, again in the middle of the back. All the temperatures recorded for adults (but not for young) were from mice which had previously experienced temperature taking three times; after the second time the mice were quite docile, even when the couple was inserted into the anus. Colon, skin and surface temperatures were all taken at between 15·00 and 17·00 hr. G.M.T.

BODY TEMPERATURES

Table 1 shows the results of measurements of temperatures. Mean group values for adult colonic temperatures vary from 37·5 to 38·1° C. Temperatures in the cold were slightly lower than in the warm; the difference is statistically significant ($P=0{\cdot}05$) for the females but not for the males. Skin and surface temperatures were markedly lower in the cold, and these differences are significant. Differences between the sexes are not significant.

Table 1 also summarizes the body temperatures found for nestlings. No significant difference was found between the sexes, and so their temperatures

Table 1. *Mean colonic, skin and surface temperatures*

(Group standard deviations in brackets)

<table>
<tr><th colspan="2" rowspan="2"></th><th colspan="2">Males</th><th colspan="2">Females</th></tr>
<tr><th>21° C.</th><th>−3° C.</th><th>21° C.</th><th>−3° C.</th></tr>
<tr><td rowspan="4">Adults</td><td>No. of mice</td><td>15</td><td>15</td><td>15</td><td>15</td></tr>
<tr><td>Colon</td><td>37·7 (1·12)</td><td>37·4 (1·10)</td><td>38·1 (1·49)</td><td>37·5 (1·54)</td></tr>
<tr><td>Skin</td><td>36·3 (1·28)</td><td>34·6 (1·54)</td><td>36·8 (0·79)</td><td>34·0 (2·00)</td></tr>
<tr><td>Surface</td><td>34·2 (1·68)</td><td>31·3 (0·55)</td><td>34·5 (0·82)</td><td>30·0 (0·63)</td></tr>
<tr><td colspan="3"></td><td colspan="2">21° C. (both sexes)</td><td>−3° C. (both sexes)</td></tr>
<tr><td rowspan="4">Young (colon only)</td><td rowspan="2">11–13 days</td><td>no. of mice</td><td colspan="2">18</td><td>39</td></tr>
<tr><td>mean temp.</td><td colspan="2">33·9 (0·51)</td><td>30·0 (2·32)</td></tr>
<tr><td rowspan="2">18–20 days</td><td>no. of mice</td><td colspan="2">26</td><td>23</td></tr>
<tr><td>mean temp,</td><td colspan="2">34·5 (2·10)</td><td>31·4 (3·87)</td></tr>
</table>

are grouped. Both in the cold and the warm the temperatures of the young were lower and more variable than those of adults. The variability was greater in the cold.

NESTS AND NEST TEMPERATURES

The form of a well-constructed nest is illustrated in Figs. 1 and 2. All mice, except those newly introduced to the cold room, build nests of this sort in the cold. In the warm room similar nests are built, but they are often open at the top, and in general they are more casually constructed. Nests of breeding pairs in both temperatures were assessed for quality of construction. 0, 1 or 2 marks were given for each of three characteristics: (*a*) degree of closure; (*b*) domed formation (cf. Fig. 2); (*c*) fluffing of cotton-wool. The maximum score was therefore six. Table 2 gives results, including some from C57 mice. The effect of the cold environment is clearly shown.

Table 2. *Nest construction*

	A		C57	
	21° C.	−3° C.	21° C.	−3° C.
No. of nests	27	61	35	19
Mean score (with S.D.)	3·6 (1·7)	5·4 (1·0)	2·4 (1·2)	5·2 (1·0)

The importance of experience of the cold is also illustrated by the behaviour of mice taken from their parents at 21 days. Those brought up in the cold immediately burrow into the cotton-wool in their new cage; while those reared in the warm, and transferred at this age to the cold room, often remain on the surface of the cotton-wool for some time.

The typical domed nest is made by manipulation of the cotton-wool with the mouth and fore-feet. The cotton-wool, initially in its usual matted state, is fluffed out: it thus comes to occupy more space, and the volume of air in its interstices is increased. The occupied nest is, however, not a stable structure: it is always liable to alteration, and disturbance may lead to the building of a new nest; this applies especially when young nestlings are present, and makes it impossible to record temperatures continuously over long periods.

Systematic study was made of nest temperatures where nestlings of 1–7 days were present. The highest nest temperature recorded was the same for each room, namely, 34·5° C. The lowest temperature, with the thermocouple among nestlings, recorded from 14 nests in the warm room was 27·5° C., that from twenty-six nests in the cold room was 20·2 °C. Nest temperatures in the cold were much more variable than those in the warm. A statement of 'average temperature', either for a single nest over a period, or for a group of nests, would, however, give only a spurious impression of precision. Owing to differences within the nest at a given moment, rapid changes of temperature with time and changes in the position of nestlings, it is not possible to make numerically precise statements of high generality about nest

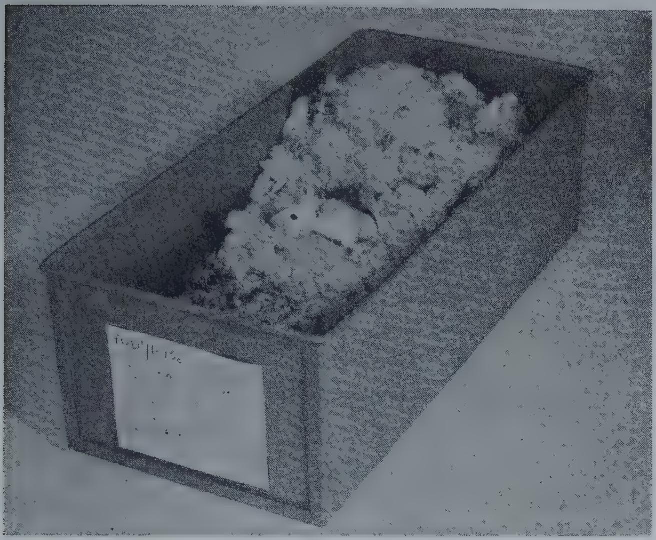

Fig. 1. Cage with lid removed, showing typical well-made nest.

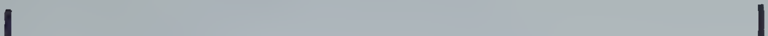

Fig. 2. Diagram of section of typical well-made nest.

temperatures. Figs. 3–6 give typical examples of the conditions in individual nests. Fig. 5, which should be compared with Fig. 6, shows how the maintenance of a high nest temperature in the cold depends on the presence of at least one adult, and the steep decline in temperature which takes place when both adults are feeding. Fig. 5 also illustrates the fact that nestlings make some contribution to the nest temperature, but not much.

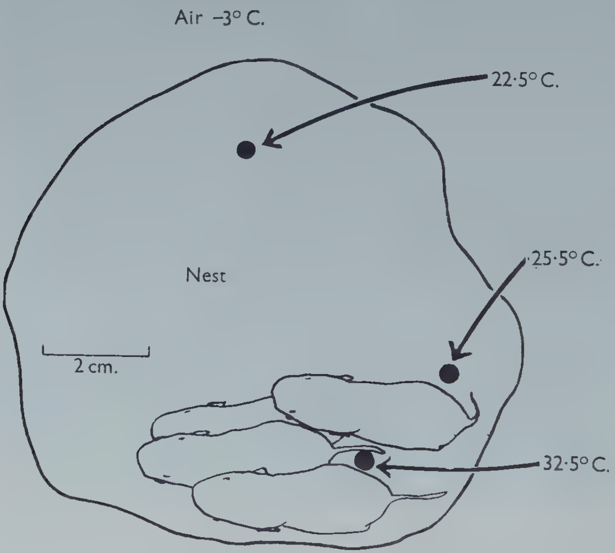

Fig. 3. Diagram of nest in cold room, showing temperature gradient.

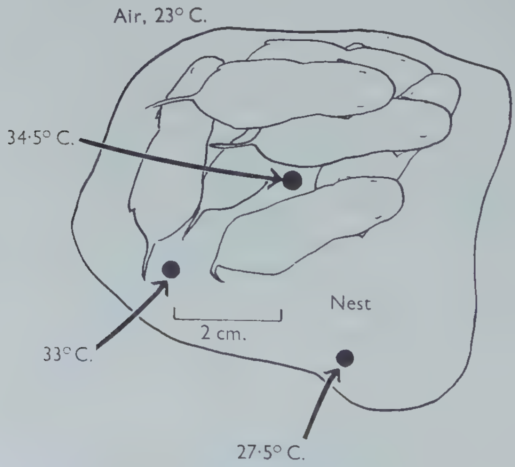

Fig. 4. Diagram of nest in warm room, showing relatively uniform temperature within nest. This nest, characteristically, was less well-made than that shown in Fig. 3.

Once the young develop hair and are able to move more actively, it becomes impracticable to record anything that can be called 'the' nest temperature. This is due partly to the disturbance resulting from the presence of the thermocouple: if a thermocouple is left in place for a time, in the hope that the mice will settle down around it, they move elsewhere.

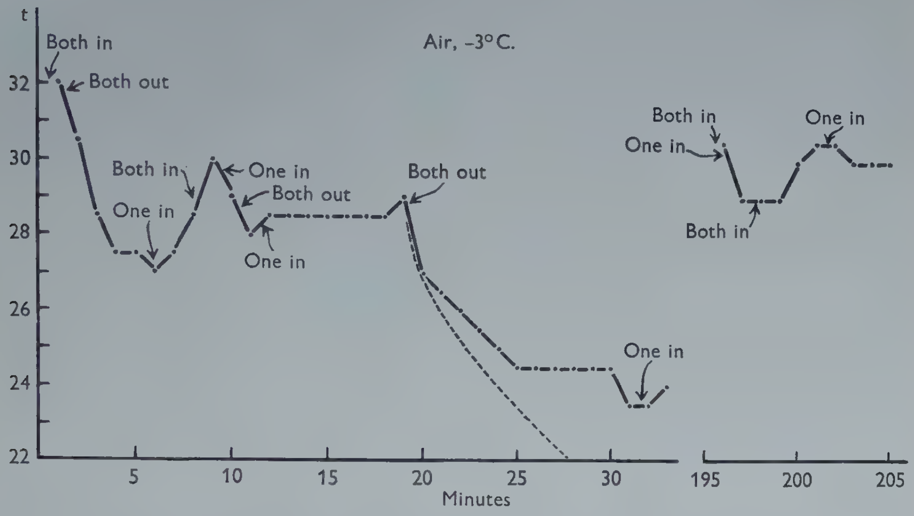

Fig. 5. Temperature fluctuations in a nest, containing a 2-day litter of five (wt. approx. 15 g.), in the cold room. All temperatures were recorded with the thermocouple on or among the litter. The effect of absence of both parents is shown. The broken line represents the decline in temperature of approximately the same weight and volume of water in a similar nest. The difference between the two curves illustrates the contribution made by the nestlings to the maintenance of nest temperature.

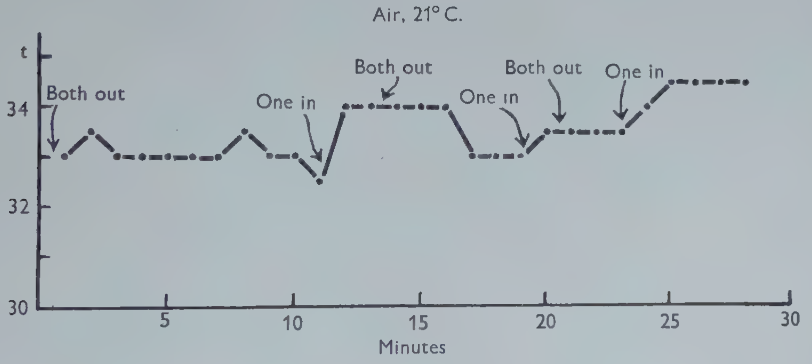

Fig. 6. Temperature fluctuations in a nest, containing a 2-day litter of eight, in the warm room. Contrast Fig. 5.

EFFECT OF NEST INSULATION ON ADULTS

To test the importance of nest material for adults, mice aged 4–12 months were subjected to the cold environment without cotton-wool. They still had sawdust on the floor of the cage, but there was not enough of this to form more than a very slight hollow where the mouse slept. Each mouse was alone in a cage. This experiment was done only with mice of the Mill Hill strain, and it is uncertain whether the Glaxo strain A mice would respond in the same way.

The results are shown in Table 3. Eleven mice which had been reared in the cold all survived exposure without cotton-wool for at least 28 days; on the 28th day one death occurred, and five more between 8 and 10 weeks. Each of these deaths was preceded by a sudden decline in weight. Apart from this decline before death, body weights remained stationary in these conditions. But, of the sixteen mice from the warm room, twelve died in the first 48 hr. Such mice, left in a *warm* room without cotton-wool, not only survive to an age of more than 2 years, but also rear litters.

Table 3. *Effect of exposure to cold without nesting material*

	No. exposed		Mean wt. g. initially (with range). Both sexes	No. dead in 48 hr.	
	♂	♀		♂	♀
Bred at −3° C.	6	5	24·8 (19·5–28·0)	0	0
Bred at 21° C.	10	6	32·3 (27·0–42·0)	8	4

DISCUSSION

Sumner (1911, 1915) studied the effects of temperatures of around 4° C. on the breeding and growth of mice. His work was never completed, and he published no physiological observations. He also had the disadvantage of having to work with a heterogeneous mouse stock. More recent studies of mammals at low temperatures have been either on the effects of acute cold stress over short periods, or on the cold resisting characteristics of animals which normally live in cold climates (reviewed by Burton & Edholm, 1955). An important exception is the work of Laurie (1946) on wild house mice living in meat cold stores at around −10° C. The present study, of which a small part is reported here, is concerned with the physiological adaptations of laboratory mice breeding for several generations at low temperatures, and with genetical variation in the response to cold. The results presented in this paper define the environmental stress to which the mice are subjected, and indicate some of the mechanisms by which the stress is withstood.

The effects of exposure to cold may include the following, among others: (i) increased mortality; (ii) altered growth of the whole body, or of relative growth of parts; (iii) changes leading to increased heat production; (iv) altered behaviour; (v) changes in later generations, e.g. through the maternal environment. All these, except the last, can be conveniently considered here.

(i) Exposure to cold undoubtedly increases mortality in mice, especially among the nestlings (cf. Barnett & Manly, 1954, 1956). Further information on the responses of young mice to cold will be published elsewhere, but it can be said that the critical period for strain A mice is the first 4–5 weeks, and that the maintenance of a relatively high nest temperature during this time is of especial importance.

This is true even though, as mentioned above, when the young mice become active nest temperatures are very unstable. Presumably, when parents and young are all asleep there is a high nest temperature, but this has not been directly observed. From the point of view of keeping up the temperature of the young, the high heat output of adults, reflected in the high surface temperature, is an advantage, since it makes possible a rapid restoration of nest temperature after the absence of the adults. High nest temperatures are, however, not the whole story. Hill (1947) has shown that infant rats are poikilothermous up to 18 days, and Fairfield (1948) found that they can survive exposure to temperatures as low as 2° C. The body temperatures reported in the present paper show that suckling mice too are thermally unstable. No doubt their ability to withstand fluctuations of body temperature is important in view of the inconstancy of the nest temperature in the cold environment.

(ii) Mice are lighter, both at weaning (Barnett & Manly, 1954) and as adults (Retzlaff, 1939), in the cold. This is probably due, in the adult, largely to the presence of less fat (unpublished), and must tend to increase the difficulty of maintaining body temperature: catabolic adaptation must cope, not only with reduced environmental temperature, but also with a body subject to a relatively higher rate of cooling. Evidently, increased food intake does not fully compensate for the additional expenditure of energy imposed by the high rate of heat loss. Changes in fur insulation might compensate for reduction in body weight. Scholander, Walters, Hock & Irving (1950) have shown that in arctic mammals insulation provided by skin and hair is an important aspect of adaptation to low temperatures. Another type of adaptive growth change, according to 'Allen's rule', might be an alteration in bodily proportions (Allen, 1877). Sumner (1915) reported that rearing mice in the cold led to a reduction in the weights and lengths of feet, ears and tails. Changes of this kind, in the mice used in the present experiments, will be reported elsewhere, but it can be said now that there is no evidence of any thermally important allometric change: relative tail lengths are slightly reduced in the cold; but, more important, the relative weight of the skin (including the hair and subcutaneous fat) is *reduced*; this again is probably due to reduction in the amount of fat in mice in the cold. It serves to emphasize that either the chemical regulation of temperature, or ectothermic control, are the important factors.

(iii) In the present study, the role of chemical regulation is most clearly shown in the different response of acclimatized and non-acclimatized mice to exposure without nest material: most of the latter failed to survive for 2 days, while those reared in the cold lived for some weeks unprotected. This is in accordance with the observations of Hart (1953*a*, *b*) on the importance of acclimatization in mice. Catabolic changes alone are, however, not enough to ensure indefinite survival, in the strain studied: some, at least, of the acclimatized mice showed a slow decline, culminating in death after some weeks.

The character of the chemical regulation has not been studied in the present experiments. Hart & Heroux (1954) have shown that, in short-term acclimatization of deer mice to cold, muscular activity, whether in the form of exercise, shivering or increased tension, must be supplemented by a passive increase in metabolic rates.

(iv) It is only when chemical regulation can be supplemented by ectothermic adjustment that normal survival is possible: that is, when a nest can be built. There is considerable scope for behavioural study of the building and use of nests in different environmental conditions. Kinder (1927) showed that albino rats make better nests if the environmental temperature is lowered, and that they improve with practice. In mice there is undoubtedly a complex interaction between innate and learned components, in the building of a nest in the cold; these remain to be studied.

In general, the present study shows that the two modes of adaptation to low temperature, the endothermic and ectothermic, are both important in the lives of mice in an environment at −3° C. It is commonly said that the success of mammals, in a wider range of niches than was previously occupied by reptiles, is due to their combination of two qualities: (*a*) a high degree of homeostatic regulation through internal changes, and (*b*) individual adaptability of behaviour in varying conditions (cf. Young, 1950). The results reported here provide a specific example supporting this generalization.

SUMMARY

Laboratory mice (*Mus musculus* L.) have been bred at an environmental temperature of −3° C., with controls at 21° C. The mice were given cotton-wool for nesting.

Body temperatures, taken in the colon, of adult control mice were about 38° C.; those of mice reared in the cold were slightly lower. Skin temperatures, taken below the hair, and temperatures on the surface of the hair, were lower in the cold than in the warm. Surface temperatures were, nevertheless, high (30° C. for females in the cold), indicating a high rate of heat loss.

Mice less than 3 weeks old have lower and more variable body temperatures than adults.

Better nests are built in the cold than in the warm. Nest temperatures, taken among nestlings less than 7 days old, are high (up to 34·5° C.) in the cold as well as in the warm; but nests and nestlings in the cold are subject to sharp declines in temperature when both parents leave the nest to feed.

Adults reared in the warm were transferred to cages in the cold without cotton-wool; most of these mice died within 48 hr. Similar mice, reared in the cold, and transferred to cages without cotton-wool, survived for some weeks, though not indefinitely. The difference reflects the state of partial metabolic (endothermic) adaptation achieved by mice reared in the cold.

The results in general show that both endothermic and ectothermic adjustments play a part in the maintenance of mice at −3° C. The ectothermic or behavioural adaptations are those involved in the efficient building and utilization of nests.

I am indebted to Prof. C. M. Yonge for the facilities provided in his department; to the Nuffield Foundation for grants in aid of the research; to Dr J. C. D. Hutchinson and Dr G. A. P. Wyllie for helpful discussions of various aspects of the work; to Margaret Doyle and Elizabeth MacMurray for technical assistance; and to John Andrew for taking the photograph.

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ON THE RELATIVE IMPORTANCE OF BODY WEIGHT AND SURFACE AREA MEASUREMENTS FOR THE PREDICTION OF THE LEVEL OF OXYGEN CONSUMPTION OF *LIGIA OCEANICA* L. AND PREPUPAE OF *DROSOPHILA MELANOGASTER* MEIG.

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(*Received* 5 *September* 1955)

INTRODUCTION

Although there is general agreement about the variation in metabolic rate with increasing body size, the reason for this variation is obscure. In general it is assumed that oxygen consumption is proportional to some function of body weight, and that the relationship of the former to surface area is fortuitous and due to the relationship of surface area itself to body weight. But measurements of the surface area of the individuals used in the oxygen determinations are almost invariably lacking for poikilotherms, and rare for other animals. In recent papers (Ellenby, 1951, 1953) the relationship of oxygen consumption to surface area and to body weight has been investigated for prepupae of *Drosophila melanogaster* and for the littoral isopod *Ligia oceanica*. For forty-seven *Drosophila* prepupae and eighty male *Ligia* information was available on body weight, and the surface area (or a linear dimension related to it) of the actual animals used in the oxygen consumption determination. The oxygen consumption per unit surface varied with weight for *Drosophila* but remained approximately constant for *Ligia*. For both animals, the material suggested that the level of oxygen consumption might be predicted more precisely from a measurement of surface area than from one of body weight, and this in spite of the greater accuracy of the weight determinations. In other words, the level of oxygen consumption might be more closely related to surface area than to body weight. In the present paper, the material is examined in greater detail in order to see how far the suggestion can be substantiated.

DROSOPHILA

The relationship of oxygen consumption to body weight appears to be approximately exponential, and it is therefore conveniently evaluated by a linear regression analysis based on the logarithms of the observations. The usual normal law assumptions appear to be valid for the transformed observations. Preliminary calculations (Ellenby, 1953) showed that the residual mean square about the regression line for log oxygen consumption on log surface area was some three-quarters of that for a regression line for log oxygen consumption on log body weight. A less valid test for *Ligia* (Ellenby, 1951) also suggested that oxygen consumption might be more closely related to surface area than to body weight. But the technique of linear regression can consider only two variables at the same time, either oxygen and body weight, or oxygen and surface area. In the present treatment the material is examined by employing the technique of multiple regression; this can consider any number of independent variables in relation to a single dependent variable and assess their relative importance. In the present case the technique is used to assess the relative importance of body weight and surface area in predicting the value of the dependent variate, oxygen consumption. As before, the logarithms of the observations are used in the analysis.

The following abbreviated notation is used throughout this section:

Log (O.C.) $= \log_{10}$ (oxygen consumption in mm.^{3} per hour at S.T.P.);
Log (B.Wt.) $= \log_{10}$ (body weight in mg.);
Log (S.A.) $= \log_{10}$ (surface area in mm.^{2}).

The values of these three variables were known for each of forty-seven *Drosophila* prepupae: thirty-three sets of values for diploid females, five for diploid males, and nine for triploid females.

Separate multiple regression equations of the form

$$\log(\text{O.C.}) = a_0 + a_1 \log(\text{B.Wt.}) + a_2 \log(\text{S.A.})$$

were fitted by least squares for each of these three homogeneous groups.

No significant differences were found between the diploid males and females, but the results for the triploid females were of a different character; the latter are therefore treated separately.

Diploid male and female Drosophila

The analysis of variance for the combined diploid male and female data is given in Table 1. The total sum of squares for log (O.C.) is 0·169 with 37 D.F. This sum of squares is partitioned into two components; the 2 D.F. sum of squares removed by fitting a multiple regression equation of the type mentioned above, and the residual sum of squares for the fitted regression equation. The table shows that $F_{2,35} = 156$, so that, as expected, there is a significant multiple regression of log (O.C.) on log (B.Wt.) and log (S.A.).

The initial part of the table is concerned with testing whether it is an advantage to have both the dependent variates log (B.Wt.) and log (S.A.) in the regression equation, or whether practically as good a prediction of oxygen consumption could be obtained by using body weight or surface area alone. The first line shows that the sum of squares removed by a linear regression of log (O.C.) on log (S.A.) is significant since $F_{1,35} = 313$. The second line shows that the extra sum of squares removed by the multiple regression of log (O.C.) on log (S.A.) and log (B.Wt.) is negligibly small since $F_{1,35} = 1/108$. (This variance ratio is not significantly small, the lower 5% point being 1/251.)

A similar procedure for log (O.C.) and log (B.Wt.) is repeated in the third and fourth lines of the table. The linear regression of log (O.C.) on log (B.Wt.) removes a significant sum of squares ($F_{1,38}=283$); however, in this case, the extra sum of squares removed by the multiple regression is also significant ($F_{1,35}=29$).

The conclusions to be drawn from this analysis are that the multiple regression equation does not give any significant reduction in the error mean square when compared with the simple linear regression of log (O.C.) on log (S.A.). The multiple regression is, however, significantly better than the linear regression of log (O.C.) on log (B.Wt.).

Table 1. *Analysis of variance for multiple regression*; Drosophila *diploid male and female data combined*

Source of variation	Sum of squares	D.F.	Mean square	F
Linear regression on log (S.A.)	0·152	1	0·152	313
Regression on the component of log (B.Wt.) orthogonal to log (S.A.)	0·000	1	0·000	0
Linear regression on log (B.Wt.)	0·138	1	0·138	283
Regression on the component of log (S.A.) orthogonal to log (B.Wt.)	0·014	1	0·014	29
Multiple regression on log (B.Wt.) and log (S.A.)	0·152	2	0·076	156
Residual	0·017	35	0·000	—
Total	0·169	37	—	—

In other words, assuming a regression of log (O.C.) on log (S.A.), a negligible increase in the precision with which log (O.C.) may be predicted is obtained by taking into account log (B.Wt.). But, on the other hand, after fitting a linear regression of log (O.C.) on log (B.Wt.), a significant improvement in the precision is obtained by taking log (S.A.) into account also. Clearly, although both log (B.Wt.) and log (S.A.) give significant information about log (O.C.), the log (S.A.) variable is the more important of the two; and this in spite of its relatively inaccurate measurement.

The simple regression line linking log (O.C.) and log (S.A.) has a slope of + 1·19, with an estimated standard error of 0·066. The slope differs significantly from unity ($P<1$ %), confirming, as already deduced indirectly (Ellenby, 1953), that oxygen consumption is not quite proportional to surface area.

Triploid female Drosophila

The individual residual mean squares for the three groups of data showed no evidence of heterogeneity; the three residual sums of squares left after fitting the three separate multiple regressions were therefore pooled. The error variance of Table 2 has been estimated from this pooled information instead of from the residual error for the triploid group alone; the effect is to give a residual mean square of 0·000480 with 38 D.F. rather than a mean square of 0·000280 with 6 D.F.

The arrangement of Table 2 is then fundamentally the same as that of Table 1. From the last two lines it is seen that $\mathrm{F}_{2,38}=38$ so that there is a significant multiple regression of log (O.C.) on log (B.Wt.) and log (S.A.). The first and second lines of the table show that the linear regression of log (O.C.) on log (S.A.) is significant ($F_{1,38}=69$), and that the extra sum of squares removed by the multiple regression of log (O.C.) on both log (S.A.) and log (B.Wt.) is also significant ($F_{1,38}=16$), that is, log (B.Wt.) contributes significantly.

Table 2. *Analysis of variance for multiple regression; triploid female data*

Source of variation	Sum of squares	D.F.	Mean square	F
Linear regression on log (S.A.)	0·029	1	0·029	60
Regression on the component of log (B.Wt.) orthogonal to log (S.A.)	0·008	1	0·008	16
Linear regression on log (B.Wt.)	0·036	1	0·036	76
Regression on the component of log (S.A.) orthogonal to log (B.Wt.)	0·000	1	0· 00	0
Multiple regression on log (B.Wt.) and log (S.A.)	0·036	2	0·018	38
Pooled residual	0·018	38	0·000	—

The middle section of the table shows that the linear regression on log (B.Wt.) is significant ($F_{1,38}=76$); however, the extra sum of squares removed by the multiple regression is now negligibly small ($F_{1,38}=1/15$), showing that log (S.A.) contributes practically no additional information about log (O.C.) beyond that already provided by log (B.Wt.).

The position, then, is almost the exact opposite of that found for the diploid males and females. A prediction of oxygen consumption for diploid males and females from surface area measurements is not improved significantly by the additional information from body weight; in the case of the triploids, however, a prediction based on body weight is not improved significantly by the additional information from surface area. For the diploids, therefore, surface area is the more important; but for the triploids it is body weight.

As already described (Ellenby, 1945), the surface area of the puparium is measured after the imago has emerged, the empty case being flattened between microscope slides. A certain number are excluded if they are badly broken in the process. In view of the scarcity of triploids, it seemed possible that they may have been judged less rigorously as one could hardly afford to lose a single one. However, comparison of the variances of the surface-area measurements for diploids and for triploids showed that they do not differ significantly ($P > 30\%$). Whatever the reason, therefore, for the difference between the triploid and diploid results it is not due to any systematic difference between the accuracy of the surface-area measurements in the two groups.

LIGIA

A plot of log length against log breadth for male *Ligia* (Ellenby, 1951) gave a straight line with a slope which did not differ significantly from unity, showing that the animals remained more or less the same shape with increasing body size; and this conclusion was also supported by an examination of the growth of the pleopods relative to body length. Since, for bodies of similar shape, surface area is proportional to the square of a linear dimension, (length)^2 was used for estimating surface area in the work already cited; and 'log length' is used in the present treatment. Multiple regression analyses were also carried out including 'breadth' but, presumably because of the larger percentage error of these measurements, this dimension was found to be very inferior to both length and weight. Consequently these analyses have not been presented here (even though the simple regression of log oxygen consumption on log breadth was highly significant), and the multiple regression analysis given below is based only on the oxygen consumption, body weight, and length (excluding the antennae and uropods) of the eighty male *L. oceanica* used in the previous work.

The following abbreviated notation is adopted:

Log (O.C.) = $\log_{10}$ (oxygen consumption in mm.^{3} per hour, at S.T.P.);
Log (B.Wt.) = $\log_{10}$ (body weight in mg.);
Log (L.) = $\log_{10}$ (length in cm.).

The analysis shows that log (B.Wt.) is the most important single variate, and that the increase in accuracy obtained by including log (L.) in a multiple regression equation is not statistically significant. That is, log (L.) is found to be relatively unimportant statistically compared with log (B.Wt.) for the prediction of log (O.C.) for male *Ligia*.

The results of the analysis are presented in Table 3. The last two lines of the table show that there is a significant multiple regression of log (O.C.) on log (B.Wt.) and log (L.). The first and second lines of the table show that the linear regression of log (O.C.) on log (B.Wt.) is significant, and that the extra sum of squares removed by the multiple regression including log (L.) is negligible ($F_{1,77} = 0{\cdot}2$); very little additional information is therefore provided by log (L.).

Table 3. *Analysis of variance for multiple regression*; Ligia oceanica *data*

Source of variation	Sum of squares	D.F.	Mean square	*F*
Linear regression log (B.Wt.)	5·44	1	5·44	699
Regression on the component of log (L.) orthogonal to log (B.Wt.)	0·00	1	0·00	0
Linear regression on log (L.)	5·38	1	5·38	692
Regression on the component of log (B.Wt.) orthogonal to log (L.)	0·06	1	0·06	8
Multiple regression on log (B.Wt.) and log (L.)	5·44	2	2·72	350
Residual	0·60	77	0·01	—
Total	6·04	79	—	—

Linear regression of log (O.C.) on log (L.) is significant, as shown in the third line of the table, with $F_{1,77}=692$; and as shown in the next line, including log (B.Wt.) gives a significant improvement in accuracy ($F_{1,77}=7\cdot8$).

Although log (B.Wt.) is the most important single variate, from our present point of view it is interesting to note that, in spite of the relative inaccuracy of the length measurements, log (L.) does not show up too badly; the residual mean square for simple linear regression of log (O.C.) on log (B.Wt.) is 0·0077 compared with 0·0085 for the residual mean square for linear regression of log (O.C.) on log (L.).

DISCUSSION

The results show, then, that for *Ligia* and triploid prepupae of *Drosophila*, body weight is more important for predicting the level of oxygen consumption than surface area: on the other hand, surface area gives a better prediction for diploid male and female prepupae.

It would ill become us to belittle the *Ligia* data which, in fact, compare favourably with that from other animals; but the conclusions drawn from the diploid *Drosophila* material and the comparison of triploids and diploids are of most interest, and these are based on material of exceptional reliability. In any case, it would be unwise to compare information based on a series of isopods differing in age and body weight with that derived from a group of insect prepupae differing in body weight but of the same age, and undergoing the cataclysm of insect metamorphosis. Compared with the *Ligia* individuals, the prepupae were genetically uniform; the measurements of oxygen consumption are based on animals of the same age, and would not have been influenced by bodily movements or digestive and related processes; body weight would also be more reliable, for the prepupae were reared under controlled conditions and weighed after many hours at constant humidity; and surface area was actually measured rather than estimated from a linear dimension. Accurate measurement of surface area is, however, extraordinarily difficult, and even a polished metal plate may have a true surface ten times its apparent surface (Bowden & Rideal, 1928); but, although the method by which puparial surface was measured is as accurate as most, it is of greater importance that there are grounds for maintaining that the measured surface bears a constant relation to the true surface over the whole size range (Ellenby, 1953). Nevertheless, as *determinations*, the surface area measurements are less accurate than those of body weight, and the finding that surface gives the better prediction of level of oxygen consumption for diploid prepupae is therefore of added significance.

The success of a prediction of the level of oxygen consumption from a measurement of body weight, or of surface area, will depend on the accuracy with which the latter are measured and on the closeness of their relationship to factors governing the level of metabolism. Clearly, even if physical surface were directly connected with the level of metabolism, it may still be possible to predict oxygen consumption with greater accuracy from body weight; for it may be possible to estimate the surface area of an individual animal more accurately from its weight than it can be measured directly. A demonstration that oxygen consumption may be predicted more accurately from body weight may only be due, therefore, to the fact that body weight can be measured more accurately.

On the other hand, although an animal may be weighed at any instant with very great accuracy, the body weight may be unreliable from the standpoint of metabolism; the chemical balance may be very trustworthy, but not the use to which it is put. For example, the water content of a particular animal may vary considerably from instant to instant, so that a prediction of oxygen consumption from wet weight is likely to be inaccurate for this reason alone. Surface area may then give a better prediction of oxygen consumption merely because it is to a large extent independent of water content, and not because it is causally related to metabolism. In other words, under these circumstances, surface area may be a better predictor than body weight because it is a more reliable measure of 'body size'.

A more accurate prediction of the level of oxygen consumption from body weight, therefore, or from surface area, does not necessarily imply a closer relation of either of these physical properties to the real factors governing the level of metabolism.

The evidence from the *Drosophila* material is therefore of particular importance in relation to this impasse: for the triploid females and the diploid males and females give different answers. It is unlikely that these different prepupae, covering the same range of body size, would show class differences in their composition of the minor sort which might operate among a collection of animals raised under a variety of nutritive conditions. Their body weights would therefore be of the same reliability as *determinations*, and the accuracy with which surface area was measured would be the same for the different classes. Yet the results show, nevertheless, that while surface area gives the better prediction for diploids, weight is better for triploid females. It would be strange indeed if this difference were not entirely because the comparison was, in fact, between diploid and triploid organisms.

Differences in the body weights of organisms may be accompanied by relative differences in their composition or their structure or in both. The superiority of body weight, therefore, may be due to a variety of causes. On the other hand, minor factors can be excluded in the case of the diploid/triploid comparison. The superiority of surface area as a standard for predicting the level of oxygen consumption of diploid male and female prepupae of *Drosophila* strongly suggests that, in this case at least, factors related to the surface itself may have a determining influence on the level of metabolism.

SUMMARY

1. Previous investigations with *Ligia oceanica* and prepupae of *Drosophila melanogaster* (Ellenby, 1951, 1953) have suggested that it may be possible to predict the level of oxygen consumption more precisely from a measurement of surface area (*Drosophila*) or body length (*Ligia*) than from body weight, in spite of the greater accuracy of the latter measurement. The point is now examined more closely by applying the technique of multiple regression to the original data.

2. For *Ligia*, it is shown that the suggestion cannot be upheld, for the level of oxygen consumption can be predicted with greater accuracy from body weight than from a function of body length.

3. On the other hand, for diploid male and female prepupae of *Drosophila*, it is shown that surface area does, in fact, give a better prediction than body weight. In the case of triploid female prepupae, however, body weight is superior.

4. It is shown that there are no grounds for believing that the measurements of surface area were less accurate in the case of the triploids; for this and other reasons, it is suggested that the difference between diploids and triploids may be due to a fundamental difference between the two sorts of prepupae.

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SOME OBSERVATIONS ON WATER-SOLUBLE FACTORS WHICH ACCELERATE THE GROWTH IN ASEPTIC CULTURE OF LARVAE OF *DROSOPHILA MELANOGASTER*

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INTRODUCTION

The desirability of having available a chemically defined medium for rearing the larval stages of *Drosophila* under sterile conditions needs no emphasis. While some progress towards such an end has been made, media at present in use are far from satisfactory. The medium suggested by Schultz, St Laurence & Newmeyer (1946), as well as those used by Hinton, Noyes & Ellis (1951), lead to excessive protraction of the larval period; that suggested by Begg & Robertson (1949), although permitting rather more rapid development, is less 'defined' in that it contains intact casein and ribonucleic acid derived from yeast. In addition, the larval period even on the latter medium is still too long for any experiments involving studies of an environment-sensitive mutation whose phenotype depends, amongst other things, on the length of the larval period.

It has from time to time been claimed (e.g. Tatum, 1939; Goldberg *et al.* 1945; Schultz *et al.* 1946) that a water-insoluble fraction derived from yeast, and containing a water-washed cell mass, is necessary for the normal development of *Drosophila* larvae. Begg & Robertson (1949) found, however, that the water soluble portion of a yeast autolysate, prepared with ether in the presence of ammonia, provides all growth requirements—as, indeed, does the fraction of that extract which is soluble in 50% ethanol. The present communication deals with some experiments on fractions derived from yeast; and with the extraction of growth-accelerating fractions from an alternative source.

MATERIALS AND METHODS

Stocks

A yellow white (y^1w) stock of *D. melanogaster* has been used in all experiments reported here. This choice was made simply because the lines of the mutant 'antennaless' in which we are ultimately interested have been derived from out-crosses with yellow-white. Flies used for oviposition in each experiment were obtained from mass matings of the progeny of parents taken in each generation from the brother-sister matings of the stock line.

Egg collection

Prior to oviposition, flies are fed in half-pint milk bottles containing the usual *Drosophila* medium to which a liberal smear of a mixture of bakers' and brewers' yeasts has been added. They remain in these bottles for 2–3 days at 18–19° C., by which time a few eggs are being laid on the surface of the medium. They are then shaken into clean empty bottles, and oviposit for 8 hr. at 26° C. on a thin layer of yeast-Fuller's earth paste (to which a trace of ethanol and acetic acid has been added) smeared on agar gel in watch glasses. It is usually possible in this way to collect from three oviposition bottles, each containing some 750 flies, as many eggs as it is wise to sterilize at one time in the apparatus described below.

Sterilization of eggs

Eggs are removed from the oviposition medium with a wash bottle, and the mixture of eggs and paste sieved as previously described (Begg & Sang, 1950). The washed eggs are stirred for half an hour in a solution containing 4% 'Cetavlon' to free them from surface debris. (Cetavlon is obtainable as a prepared concentrate from Imperial Chemical Industries in Britain. In U.S.A. a satisfactory substitute is 'Cetylamine'. Both are used as a 20% solution of the concentrate in water.) The eggs are now dechorionated by running over them a 1% filtered solution of calcium hypochlorite for half an hour in the apparatus shown in Fig. 1A. They are then transferred to the chamber marked (*a*) in Fig. 2. All solutions used from this stage onwards have been autoclaved, and the operations are carried out under a dust-free hood previously exposed to ultra-violet light. Dechorionated eggs are then sterilized as follows:

Air is delivered from a small variable stroke pump (running at 4 r.p.m.) to the sterile filter tube (*b*, Fig. 2) via a Bunsen slit valve arranged to prevent return of air to the pump. Thence, via the second valve, it forces a sterile 4% 'Cetavlon' solution over the eggs in (*a*) from the Woulff bottle (*c*), and eventually into a sterile cotton-stoppered receiving flask. As fluid passes, the eggs in (*a*) are carried to the upper of the two 100 mesh/in. platinum grids which guard the ends of the standard cone and socket Pyrex joints forming the chamber. During the return stroke of the pump, when fluid has stopped moving, the eggs cascade to the bottom of the chamber, and the cycle is repeated. Thus in each cycle eggs are thoroughly but gently separated, agitated and washed with fresh fluid. This washing is continued for half an hour, by which time some 400 ml. solution should have been passed over the eggs. (*b*) and (*d*) are now transferred to a second Woulff bottle, this time containing sterile water, and washed as before for a further half hour; after which (*a*) is detached from the standard cone and placed, with the eggs, in a tube of 70% sterile (refluxed) ethanol. The eggs are at once pipetted into a test-tube containing a little 70% ethanol, and soaked for 20 min. Finally, they are pipetted, with the usual aseptic precautions, on to carmine-stained sterile agar slabs, preferably prepared in vessels made from standard Pyrex joints as indicated in Fig. 1B.

There they hatch over 24 hr. at 25° C., and larvae are picked off and put on media with platinum spoons in the usual manner. Alternatively, eggs may be transferred directly to culture tubes in a similar way. Larvae are usually used at a density of 25/culture, eggs 40–50/culture.

In laboratories where the normal room temperature is around 25° C. rather than 18° C., sterilizing times should be reduced by about 25 %.

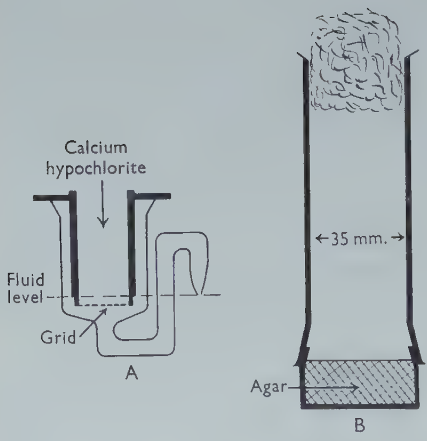

Fig. 1

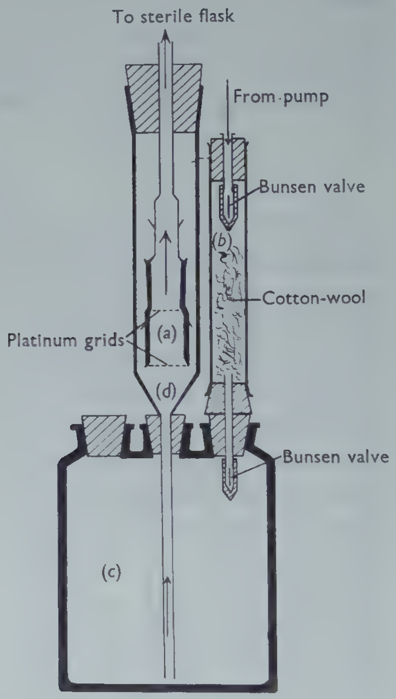

Fig. 2

Fig. 1. A. Dechorionating chamber. Eggs lie on grid indicated by dots. Fluid level when in operation indicated by dashes. Inner chamber is removable, and made of brass with phosphor-bronze grid. B. Convenient vessel for hatching of sterilized eggs.

Fig. 2. Apparatus for sterilization of *Drosophila* eggs.

Using the above technique, infected cultures are very exceptional. Nevertheless, all cultures are carefully checked for infection by staining smears from each tube with malachite green, and by subculture where deemed advisable. It is true that a fraction of eggs is killed by the somewhat harsh and prolonged procedures employed; but any less thorough treatment produces a substantial proportion of infected cultures. The loss of eggs during sterilization is not a great handicap, since large numbers can be collected and treated at one time.

Media

The basal medium used in the experiments here described was essentially that of Begg & Robertson (1949). For convenience of reference, its composition is given below.

Basal medium

	g.		μg./culture		mg./culture
Casein	6	Biotin	0·2	K_2HPO_4	2·5
DL-Tryptophane	0·5	Folic acid	4·0	KH_2PO_4	2·5
Ergosterol	0·5	Choline chloride	1000	$MgSO_4$	1·0
Cholesterol	0·5	Aneurin hydrochloride	4·0	$MnSO_4$	0·5
D-Glucose	10	Pyridoxin	12·0	NaCl	0·5
Ribose nucleic acid (from yeast)	0·3	Ca-pantothenate	12·0	$FeSO_4$	0·5
Agar (use 0·35 g./culture)	10	Riboflavin	6·0	$CaCl_2$	0·5
		Nicotinic acid	12·0		
		Thymine	25·0		

Total fluid/culture = 5 ml.

It is perhaps worth remarking in this context that experiments in which additional nitrogen sources were fed in the form of other proteins (e.g. casein, lactalbumen) revealed no significant acceleration in growth (see Table 1). When, however, a medium becomes available which allows more rapid development than the one currently used, it may be necessary to reinvestigate the whole problem of the source and balance of amino acids in the medium.

Table 1

Medium	Mean larval period (days)
Basal only	9·8
Basal + 0·1 g. extra casein/culture	9·8
Basal + 0·3 g. extra casein/culture	9·3

All extracts added to cultures were brought to pH 6·7–7·0 before use. Each extract has been tested against controls set up at the same time from the same group of eggs, and prepared from the same sample of basal medium. Thus differences in developmental time due to changing sterilizing procedures or incubators, or to the continued close inbreeding of the stock, are minimized as sources of error.

Scoring of results

Since we are primarily interested in the duration of the larval period, the mean time from larval hatching to pupation would be the ideal measure of the effectiveness of various extracts. However, in earlier experiments eggs and not larvae were put into cultures. Where eggs were placed in cultures after sterilization, the mean time in days from sterilization to pupation is given. Where eggs were allowed to hatch and larvae then picked off and put in cultures, the mean larval period is given. The error introduced by observing pupae instead of adults is unimportant, since the pupal period is virtually constant and independent of the larval period.

The position of each pupa is marked in ink on the wall of the tube as it is counted, and the final result expressed as a graph whose ordinate is the percentage of all pupae formed which appeared on the day indicated on the abscissa.

EXPERIMENTAL

Since a minority of the experiments reported here was carried out with fractions derived from yeast, only a summary of results obtained will be given.

Yeast autolysate was prepared as described by Begg & Robertson (1949). The portion of this autolysate soluble in 50% ethanol allows normal development of larvae when added to basal media in a concentration of 300 mg./culture.

Extraction of an aqueous solution of the 50% ethanol fraction with *N*-butyl alcohol for 20 hr. in a liquid extractor leaves an active aqueous phase. Similarly, extraction of vacuum-dried material with aqueous (90%) pyridine fails to remove the activity. Dialysis against running water, however, leaves a fraction in the dialysis bag which is quite inactive. Attempts to fractionate the autolysate with heavy metals were unsuccessful. Fractional precipitation with alcohol or acetone did not separate the material clearly into active and inactive components. Column treatment with 'decalso', although indicating some loss of activity in the effluent, was inconclusive.

Begg & Robertson (1949) described a fraction prepared by extracting fresh bakers' yeast with 2% sodium bicarbonate, and precipitating the filtrate from such an extraction with hydrochloric acid and ethanol. This precipitate accelerated larval growth on basal media and did not lose activity when extracted with ethanol or ether. When, however, such a precipitate is freed, by repeated reprecipitation, from ninhydrin-positive material, the activity is lost. Comparison of the bicarbonate fraction with a commercial yeast nucleic acid preparation (which is quite inactive) by means of a quantitative Bial reaction (Mejbaum, 1939) indicated only a small amount of contaminating material. The implication is that, whatever produces the acceleration noted with bicarbonate fraction is needed in rather small amounts, and is not ribonucleic acid.

In the course of experiments on the protein-containing fractions from preparations such as the above, a tryptic digest was made. The trypsin preparation used was a commercial powder. The results of an experiment with such digested fractions indicated that cultures receiving the trypsin powder alone grew as well as those receiving undigested or digested bicarbonate fractions.

Since preparations from yeast have disadvantages associated with filtration difficulties, it seemed worth while to investigate the possibility of using a trypsin powder as an alternative starting material. The remainder of this communication describes some properties of this material.

Fractions derived from pancreas

5 g. 'trypsin' powder were autoclaved at 15 lb. for 2 hr. with 0·01 N-hydrochloric acid. Unhydrolysed protein was filtered off, and 2 ml. of the clear brown neutralized extract added to each tube of basal medium. Results are plotted in Fig. 3. It is clear that such a fraction accelerates development significantly. Similar results are obtained if whole powder is added instead of the slightly acid extract (50 mg./culture).

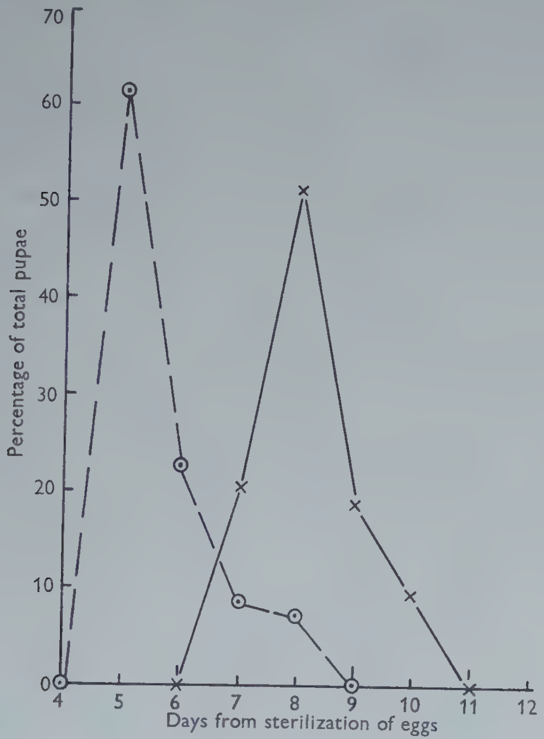

Fig. 3. ×——× basal medium only. ⊙— —⊙ basal + 0·01 N-HCl extract of commercial tryptic powder.

To determine whether the acceleration in growth was due to trypsin as such, a crystalline trypsin preparation in a concentration of 50 mg./culture was used. No effect on growth rate was observed; so it may be concluded that the factor(s) responsible for the effect on growth is present as an impurity in the commercial product.

In view of this finding, it became important to know whether all commercial trypsin powders would give similar results. A second sample was therefore obtained and tested, and found to be inactive in producing more rapid development. This confirmed that the activity did not reside in trypsin as such, but in some component which could be isolated from pancreas at the same time during its commercial production. It also suggested the desirability of preparing fractions from raw pancreas, rather than using commercial powders; a method of doing so is described later.

Fractionation of the acid filtrate from 'active' trypsin powder with 85 % ethanol gave a residue and a filtrate both of which showed some activity; combined, the activity of whole extract was restored (Fig. 4). This accords with earlier findings for yeast autolysate.

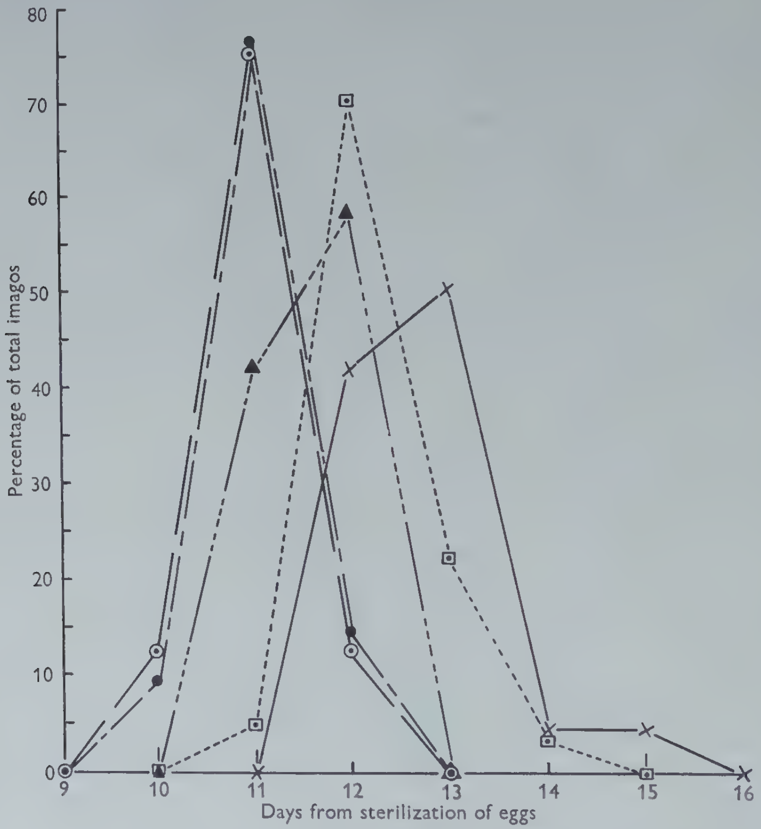

Fig. 4. ×——× basal medium only. ⊙——⊙ whole commercial tryptic powder+basal. ⊡----⊡ 85 % ethanol precipitate from 0·01 N-HCl extract of powder+basal. ▲------▲ 85 % ethanol filtrate from 0·01 N-HCl extract of powder+basal. ●----● 85 % ethanol precipitate filtrate+basal.

Passage of the slightly acid extract through a 15 × 1·3 cm. column of a cation exchange resin (Amberlite 120(H)) yielded an active effluent. However, when such an effluent is passed through an anion exchange column (Amberlite 400(OH)) some of the activity is removed (Table 2). There is some evidence here that we may be dealing with two components. Further data relevant to this possibility will be presented later.

At this stage, in view of the uncertainty of being able to obtain a constant supply of trypsin preparations with the desired properties from commercial sources, a preparation was made with fresh pig pancreas. Most of the fat was cut from 1·5 kg. of fresh pancreas. The remaining tissue was minced thoroughly, stirred with 25% ethanol (1500 ml.), squeezed through muslin, and the turbid filtrate dried down *in vacuo*. The slightly moist product was treated with a large excess of acetone, filtered on a Buchner, washed with acetone and ether, dried *in vacuo*, and finally ground to a fine powder and stored in an airtight container. From this material, soluble extracts were then prepared as required by autoclaving a 1% suspension of the powder with 0·01 N-hydrochloric acid for 15 min. at 15 lb., filtering, and retaining the filtrate. A typical experiment in which 1 ml. of a preparation of this kind was added per culture is shown in Fig. 5.

Table 2

Medium	Mean time from sterilization to pupation (days)
Basal only	11·0±0·1
Basal+0·01 N-HCl extract of trypsin powder	9·1±0·1
Basal+effluent from cation followed by anion exchange	9·8±0·2

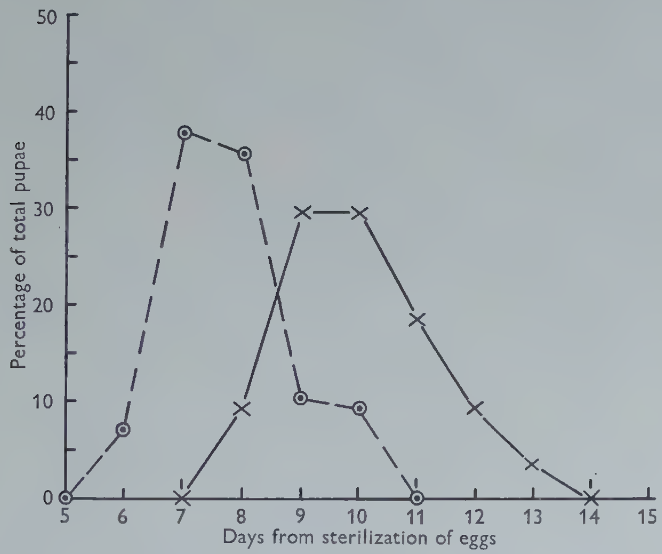

Fig. 5. ×——× basal medium only. ⊙— —⊙ basal+0·01 N-HCl extract of pig pancreas powder.

100 ml. of such a filtrate was passed over a cation column as described above. 50 ml. of the effluent were then run through Amberlite IRA 400(OH) in a 50 ml./ burette. The effluent was retained, and the water-rinsed column eluted slowly with a large volume of 5% ammonia. Those fractions of the eluate giving a ninhydrin positive reaction were combined, concentrated and tested for activity, as were the

fractions which were ninhydrin-negative. The results are shown in Table 3. These results suggest that the effluent from the cation exchange column is still active, but that some activity is removed by the strong-base anion exchanger. An approximately equivalent amount of activity is recoverable by elution. While nothing but a crude separation can be expected when exchange and elution are carried out *en masse* as here, it is perhaps significant that some activity is recoverable from the ninhydrin-negative portion of the eluate, as well as from the ninhydrin-positive fraction. This point will be returned to in the discussion; attempts at chromatographic ion exchange separation are now in progress.

Table 3

Medium	Mean time for sterilization to pupation (days)
Basal only	10·5±0·2
Basal+effluent from cation exchange	7·9±0·2
Basal+effluent from anion exchange	9·4±0·2
Basal+ammonia eluate (ninhydrin positive)	9·4±0·1
Basal+ammonia eluate (ninhydrin negative)	9·4±0·1

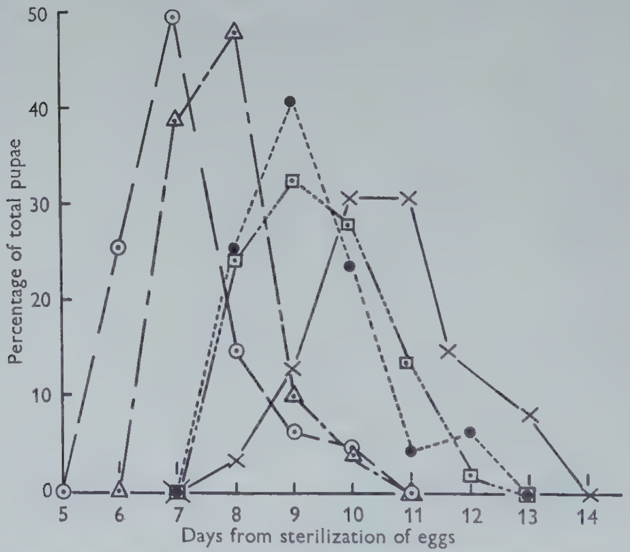

Fig. 6. ×——× basal medium only. ⊙— —⊙ 0·01 N-HCl extract+basal. ●- - - -● effluent from anion exchange column+basal. ⊡— – – —⊡ ammonia eluate of anion exchange column +basal. △— – —△ effluent+eluate+basal.

A similar experiment in which cation adsorption was omitted is shown in Fig. 6. As may be seen, anion adsorption again removes some activity, this activity being at any rate partially recoverable on elution with ammonia. Table 4 shows for another experiment the results of combining the effluent from anion adsorption with an eluate using 20% ammonia.

An experiment using charcoal (Norite A) as adsorbent indicated again that two components may be present. 70 ml. pancreatic extract prepared in the usual manner were shaken with 1 g. Norite A. The filtrate from this was shaken with 2 g. Norite, and the process repeated once more. 1 ml. of the final filtrate was added to each culture. Results are tabulated in Table 5 and plotted in Fig. 7. It is clear

Table 4

Medium	Mean time from sterilization to pupation (days)
Basal only	10·5 ± 0·1
Basal + anion effluent ammonia eluate	8·9 ± 0·1

Table 5

Medium	Mean time from sterilization to pupation (days)
Basal only	9·2 ± 0·09
Basal + charcoal filtrate	8·4 ± 0·07
Basal + 0·01 N-HCl extract	6·9 ± 0·05

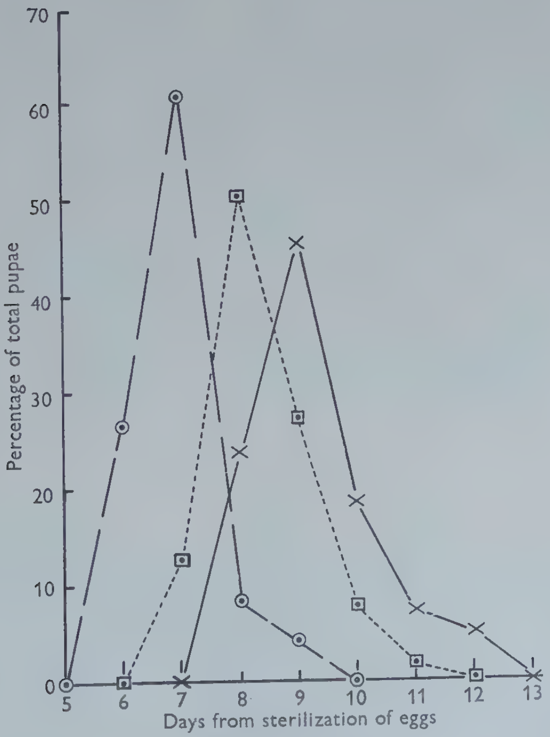

Fig. 7. ×——× basal medium only. ⊙– – —⊙ basal + 0·01 N-HCl extract of pig pancreas powder. ⊡– – – –⊡ basal + filtrate from charcoal adsorption of HCl extract.

that some activity remains even after such treatment, and evidence has been obtained that elution of the combined charcoal residues with ammonia produces a partially active eluate.

As was mentioned earlier, the ability of the soluble products of yeast autolysis to accelerate the growth of larvae is lost on dialysis against water. That the active portion of extracts of pancreas is also dialysable is indicated by Fig. 8 and Table 6.

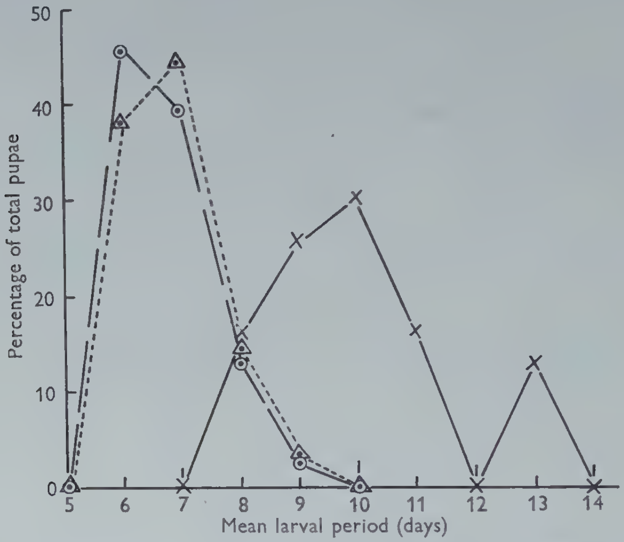

Fig. 8. ×——× basal only. ⊙— —⊙ 0·01 N-HCl extract of pig pancreas powder + basal. △----△ dialysate of HCl extract + basal.

Table 6

Medium	Mean larval period (days)
Basal only	9·7 ± 0·3
Basal + 0·01 N-HCl extract	6·7 ± 0·1
Basal + dialysate of 0·01 N-HCl extract	6·8 ± 0·1

DISCUSSION

The preliminary experiments described here confirm that larvae of *D. melanogaster* can be reared successfully in aseptic culture on water-soluble components. It is of some interest that the additional factors necessary to accelerate growth on basal media can be obtained in water-soluble, heat-stable form from sources as divergent as yeast and mammalian tissue. There has existed, and indeed still exists, the possibility that the factor or factors concerned may be no more than some amino acid essential to this insect. However, the probability that this is so is minimized by the finding that the addition of various other proteins (e.g. gelatin, globin from haemoglobin, crystalline trypsin, lactalbumen) does not accelerate growth, although some effect on pupal and adult size is sometimes observed. Again, there is the possibility that amino acid balance might be important; and that, in adding various fractions to basal media, we might in fact be making readily available to the animals some amino acid(s) they require in particularly large amounts. While it is technically impracticable to test all amino acids in all proportions, work on amino acid requirements in mammals suggests that such an hypothesis is unlikely. Further, some acceleration in growth is noted even in ninhydrin-negative fractions (Table 3), suggesting that at least one component is not such a compound.*

As regards the non-protein components of the basal medium, it is worth remarking that, while Sang's work (personal communication) suggests that at any rate under some circumstances there are *optimum* (as distinct from *sufficient*) concentrations for these; the present writer's experience has been that variations in the concentration of vitamins has little effect except when quantities are reduced below threshold values.

On the other hand, the possibility that a peptide may be involved is by no means excluded. The fact that part of the activity of pancreatic extracts remains after adsorption on charcoal is perhaps suggestive in this respect. That such molecules are present in our extracts is clear from the increase in total ninhydrin-reactive material observed when extracts are hydrolysed, as determined by the titrimetric method of Van Slyke, MacFadyen & Hamilton (1941); and by the fact that two-dimensional paper chromatograms, using phenol and *N*-butanol as solvents, reveal the absence of a ninhydrin-positive spot present before hydrolysis (R_F in phenol$\simeq$ 0·73, butanol$\simeq$0·35). It is also of interest in this connexion that similarly conducted chromatograms of the partially active effluent from adsorption on the anion exchange resin Amberlite 400 shows a spot in a similar position, and no other significant amount of ninhydrin-positive material. The idea that small peptides may be relevant as growth factors is not new (cf. Sprince & Woolley, 1945), and cannot be neglected in such studies. However, no certainty as to the nature of the factor(s) concerned can be claimed at present. The few properties so far noted give hope that separation of the components present in the active dialysate of pancreatic extracts may be possible using ion exchange and paper chromatography. Present work is concentrated on this aspect.

SUMMARY

1. An effective technique is described for the aseptic culture of larvae of *Drosophila melanogaster*.

2. Factors which accelerate the growth of *Drosophila* in aseptic culture on a basal medium containing casein as a source of amino acids can be extracted from both yeast and pancreas in water-soluble form.

* *Note added in press.* Further evidence suggesting that the growth factor is not an amino acid has recently been obtained as follows. An active pancreas fraction suspended in dry acetone was treated with DL-camphor-10-sulphonic acid, and the acetone-soluble amino-acid derivatives so formed removed by filtration. Subsequent treatment with dry NH_3 yields the free amino acids (method of McCollum & Rider (1952) *Arch. Biochem. Biophys.* **40**, 20). Such a precipitate, added at the rate of 30 mg./culture, had no effect on development time.

3. Part of the activity of such fractions can be removed by adsorption on anion exchange resins, or on charcoal; and eluted with ammonia.

4. The whole activity is dialysable and recoverable from the dialysate by evaporation under reduced pressure.

5. The active components are heat stable.

6. The active fraction is insoluble in *N*-butanol, ethanol, acetone, pyridine and ether.

7. Some evidence is presented for the presence of at least two components.

8. The possibility that one of the factors may be a peptide is considered. It is thought unlikely that it is an amino acid.

I wish to express my thanks to the Carnegie Trust for the Scottish Universities for a grant in aid of equipment; to the Rockefeller Foundation of New York for a Fellowship Grant in 1955; to the California Institute of Technology for their hospitality during that year; to the University of Aberdeen for leave of absence; to Dr James H. Sang of the Poultry Research Centre in Edinburgh for frequent helpful discussions; and to Miss Linda Roberton for technical assistance.

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THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

ACTION OF pH, DINITROPHENOL, DINITROPHENOL + VERSENE, AND USNIC ACID ON O_2 UPTAKE

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INTRODUCTION

Since Gray showed in 1928 that dilute suspensions of sea-urchin spermatozoa respire at a higher rate, per spermatozoon, than dense suspensions, evidence has been accumulating which suggests that sea-urchin spermatozoa are in different physiological conditions in suspensions of different densities. This paper is concerned with these differences and, in particular, with the effects of rigorous pH control on them. The expression 'different physiological conditions' has had a deeper significance in regard to sea-urchin spermatozoa than it would have if used to describe differences between dense and dilute suspensions of mammalian spermatozoa. In the latter, dilution at 37° C. causes irreversible damage of a pathological nature. Two examples of the alleged physiological differences between dense and dilute sea-urchin sperm suspensions are: (1) Nelson (1948) stated that usnic acid stimulated the respiration of dense suspensions ($4{\cdot}1 \times 10^8$–$1{\cdot}12 \times 10^9$ spermatozoa/ml.)* by as much as 660 %, but totally inhibited the respiration of dilute suspensions (10^8–$1{\cdot}8 \times 10^8$/ml.); (2) Rothschild (1948) reported that the inhibition of respiration by CO in the dark varied inversely with the density of the sperm suspension. Usnic acid is an antibiotic derived from lichens (Marshak, 1947); it inhibits the cleavage of sea-urchin eggs and their phosphorus uptake (Marshak & Harting, 1948), and stops the fusion of the male and female pronuclei (Marshak, 1949). Usnic acid also uncouples oxidation and phosphorylation (Johnson, Feldott & Lardy, 1950). Nelson's observations suggest the possibility that in dilute suspensions of sea-urchin spermatozoa phosphorylation and oxidation are to a certain extent uncoupled and that usnic acid may, therefore, not stimulate their oxidative processes; but that this is not so in dense suspensions, in which case usnic acid could cause an increase in their O_2 uptake. Bearing in mind the insolubility of usnic acid in sea water and the problem of the permeability of intact cells to such substances, it seemed reasonable to test this hypothesis of 'uncoupling' by examining the effect of 2,4-dinitrophenol (DNP) on the O_2 uptake of dense and dilute suspensions. Such experiments are described in this paper. Tyler & Horowitz (1937*a*, 1938) showed that the stimulation of oxidation and the inhibition of cleavage by DNP in marine eggs is a function of the concentration of the undissociated molecule in the surrounding solution. At pH 7·30, the concentration of DNP (pK 4) necessary to block cleavage was found to be about $1{\cdot}3 \times 10^{-7}$ M, while at pH 7·92 it was $8{\cdot}8 \times 10^{-7}$ M. The amounts of undissociated DNP in solution are approximately the same at these two concentrations and pH's. A prerequisite of DNP experiments on sea-urchin spermatozoa is, therefore, a knowledge of the pH of the medium and any changes in pH which may occur as a result of shaking the suspensions in manometer flasks containing KOH. Unfortunately, experiments on these subjects show that interpretation of previous results in which little or no attention was paid to pH is, to say the least, difficult. A review of these previous results follows, but it is convenient first to recapitulate the respirometric phenomena which have been observed in suspensions of sea-urchin spermatozoa in sea water, in graphical form.* Fig. 1 *a* shows the rate of O_2 uptake of a dense and a dilute suspension in sea water; Fig. 1 *b* the effect of dilution with sea water during the experiment, on the O_2 uptake of a dense solution; and Fig. 1 *c*, the effect, on the O_2 uptake of a dense suspension, of dilution before insertion into the manometer flasks. The decline in the O_2 uptake of a dense suspension at the beginning of the experiment and the gradual change-over to a more constant rate can be seen in Fig. 1 *a*. Figs. 1 *b* and *c* show the Dilution Effect, the phenomenon apparently being more marked if the dense suspension is allowed to age before dilution. Fig. 1 *c* does not support this inference, as dilution before the start of the manometric experiment involves less ageing than the experiments in Fig. 1 *b*. Provided O_2 saturation is adequate, no other differences are observed between untreated dense and dilute suspensions in sea water, except that the spermatozoa die much more quickly in dilute suspensions, as in the case of mammalian spermatozoa.

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* Throughout this paper '$x \times 10^8$/ml.' means '$x \times 10^8$ spermatozoa/ml'.

The results of metabolic studies on spermatozoa are nowadays expressed in terms of the number of spermatozoa per ml. or μl. of suspension, the number being estimated with a haemocytometer or photoelectric absorptiometer after calibration with a haemocytometer. In the past, other methods of quantifying results have been used. Gray (1928) expressed O_2 uptake in terms of the amount of nitrogen in his suspensions, but this does not distinguish between nitrogen in spermatozoa and in seminal plasma. As the ratio N(spermatozoa)/N(seminal plasma) is about 20/1 in the semen of *Echinus esculentus* (which contains about 2×10^{10} spermatozoa and 70 mg. N per ml.) and there are large variations in the number of spermatozoa per unit volume of semen from different sea-urchins, comparisons of different experiments by reference to the nitrogen content of the suspensions can be misleading. Barron, Gasvoda & Flood (1949), using *Arbacia punctulata*, estimated the dry weight of the spermatozoa used in their experiments. Their procedure was to dilute the semen with sea water; centrifuge; discard the supernatant sea water + seminal plasma; take a subsample of the packed spermatozoa for dry-weight estimation; and resuspend the sample in sea water for experiments. It is not easy to understand these experiments in terms of numbers of spermatozoa per ml. of suspension, and no details of manometric procedures are given. Moreover, the paper contains some curious statements, for example, that 'sperm diluted to 1 : 1600' (p. 45) exhibit no measurable respiration, and that this was not due to the insensitivity of the apparatus. If this means that the semen was diluted 1/1600 with sea water, the sperm density of the suspension was about 10^7/ml., in which case the spermatozoa would be highly motile and therefore expending energy. Barron and his co-workers were, however, the first to make a quantitative examination of the Dilution Effect. They found a maximum Q_{O_2} of about 20, temperature not stated, at a dilution of 1/200, i.e. at a concentration possibly of 10^8/ml.* A Q_{O_2} of 20 is almost certainly too low and due to damage following centrifugation and removal of seminal plasma before the measurements. At 15° C., probably a lower temperature than that used by

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* Further experimental details will be found under Material and Methods.

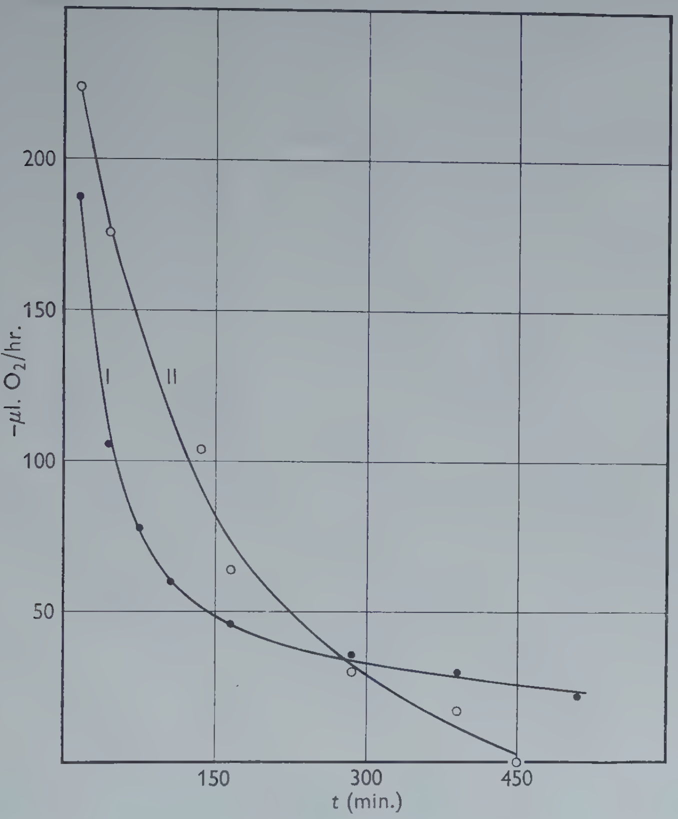

Fig. 1*a*.

Fig. 1*a*. Rate of O_2 uptake, per unit number of spermatozoa, of two suspensions of sea-urchin spermatozoa in sea water. Semen diluted in manometer vessels. I, $1{\cdot}48 \times 10^9$ spermatozoa in 2 ml.; II, $7{\cdot}40 \times 10^8$ spermatozoa in 2 ml. The following characteristics of the respiratory Dilution Effect can be seen: (1) From 0 to 60 min. the rate of decline in O_2 uptake is greater in I than II. (2) At $t=45$ the rate of O_2 uptake in II is nearly double that in I. (3) In I the rate of decline in O_2 uptake markedly slows up at about $t=150$ and is small at $t=450$, when the O_2 uptake of II is zero and all the spermatozoa are dead.

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* Dr E. B. Harvey told me that the semen of *A. punctulata* contains about 2×10^{13} spermatozoa/ml.

Barron *et al.* (1949), the Q_{O_2} of spermatozoa of *E. esculentus* may be more than 30 (Rothschild, 1950). The methods of expressing the results of metabolic experiments on sea-urchin spermatozoa have been discussed in some detail because their high Q_{O_2} makes it necessary to establish rather than assume that O_2 saturation is adequate in manometric experiments; and because the pH of the usual suspending medium, sea water, varies greatly with the density of the spermatozoa. As regards

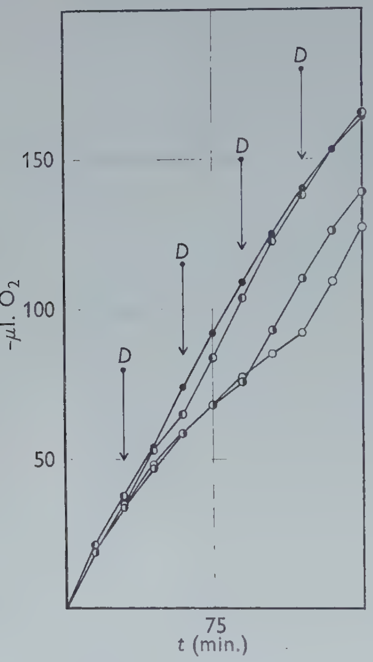

Fig. 1*b*

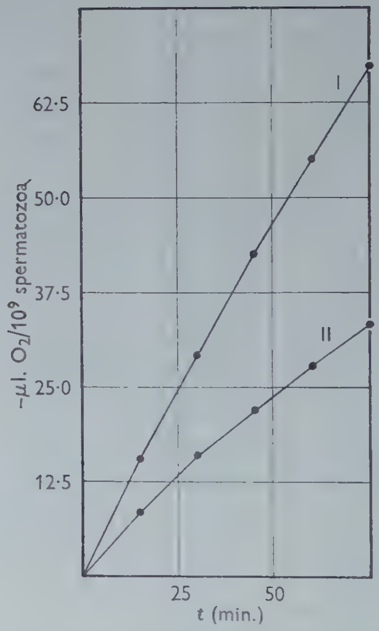

Fig. 1*c*

Fig. 1*b*. O_2 uptake of sea-urchin spermatozoa in sea water. Four sperm suspensions, each consisting of 1 ml. containing $8{\cdot}19 \times 10^8$ spermatozoa (original dilution of semen 1/30), were diluted by addition of 1 ml. sea water at times indicated by *D*. The respiratory Dilution Effect is more marked when the rate of O_2 uptake has declined, or after a certain period of incubation.

Fig. 1*c*. O_2 uptake of sea-urchin spermatozoa in sea water, the dilutions having been carried out before the beginning of the experiment. I, 2 ml. of semen diluted 1/50 with sea water, $4{\cdot}9 \times 10^8$/ml. II, 2 ml. of semen diluted 1/25 with sea water, $9{\cdot}8 \times 10^8$/ml.

O_2 saturation, if one puts 2 ml. of a suspension containing 5×10^9/ml. into an ordinary Warburg flask which is shaken at 95 c./m., and if, after the usual equilibration period and 0–10 min. reading, one goes through the motions of tipping a (non-existent) substance from a side-arm into the suspension, a sharp rise in O_2 uptake is often observed. The explanation is as follows: at this sperm density the spermatozoa in the lower layers of the suspension do not get enough O_2 for normal respiration. When, however, the 'tipping' operation is carried out, the manipulation of the manometer agitates the contents of the flask, particularly if the side-arm is washed out, much more than the usual shaking. Consequently O_2 saturation is temporarily achieved, and an increase in O_2 uptake, followed by a roughly exponential decline, is observed. These artifacts are more noticeable with modern 'smooth' shaking mechanisms than with the old-fashioned pulley reciprocating-bar type of shaker. As substances whose effects on metabolism are to be studied are often added from side-arms, the results of experiments on sea-urchin spermatozoa in which no details of sperm densities, shaker speeds, or O_2 saturation checks are given should not be accepted until and unless they are repeated under rigorously defined conditions.

The existence of physiological differences between sea-urchin spermatozoa in dense and dilute suspensions has also been claimed in the following papers:

(1) Barron & Goldinger (1941) reported that iodoacetate and malonate stimulated the respiration of sea-urchin spermatozoa, species not stated, the effect being greater in dense than in dilute suspensions. No details about sperm densities or experimental conditions are given. (The authors also said that succinate increased the respiration of sea-urchin spermatozoa and was oxidized by them.) Though these observations may be correct, they should not be accepted without confirmation. Barron & Goldinger's interpretation of these results, that —SH groups in the seminal plasma inhibit sperm respiration and combination of these —SH groups with iodoacetate reduces the inhibition, thereby allowing the spermatozoa to respire at a higher rate, is incorrect; because the addition of seminal plasma to sea-urchin spermatozoa does not inhibit their respiration (Rothschild, 1950).

(2) Barron, Seegmiller, Mendes & Narahara (1948) found that nitrogen mustards increased the respiration of sea-urchin spermatozoa (*A. punctulata*), there being an induction period of some 30 min. before the stimulation of O_2 uptake occurred. They say, p. 270, that 'the results seem to depend or vary with the concentration of sperm'. At a dilution of 1 part by volume of washed spermatozoa in 200 parts of sea water, 0·001 M methyl bis (β-chloroethyl) amine HCl stimulated O_2 uptake by 120% in the first hour after addition, 54% in the second hour, 0% in the third hour and −27% (i.e. inhibition of O_2 uptake) in the fourth hour. The O_2 uptake of control suspensions was said to be constant for 9 hr. No pH measurements were made, but there is no doubt that this substance has an effect on sea-urchin spermatozoa. The question of different effects according to sperm density requires clarification. The authors made some observations on the R.Q. of sea-urchin spermatozoa, but as the measurements were made by the direct method, when there may be a pH difference of as much as 1 between the flasks containing and not containing KOH, their results should not be accepted. In addition, tissues may respire at different rates in the presence and comparative absence of bicarbonate in the external medium (Laser, 1935).

(3) Rothschild (1948) described an experiment on the O_2 uptake of suspensions of sea-urchin spermatozoa (*E. esculentus*), in which the evolved CO_2 was absorbed by KOH and in which the suspending medium, sea water, was acidified before the experiment. Referring to the small differences between the O_2 uptake of suspensions which, at the *start* of the experiment, were at different pH's, he said (p. 357), 'This experiment disposes of the possibility that pH changes are responsible for the

Dilution Effect.' This statement is wrong, because the experiment does not enable a distinction to be made between (*a*) a reduction in pH having no effect on sperm respiration, and (*b*) a *temporary* reduction in pH, which disappears to a greater or lesser extent during the experiment, having no effect on sperm respiration. The addition of acid to sea water without any precautions to expedite the removal of CO_2 can give misleading results as regards pH.

In the same paper Rothschild said that the hypothesis of CO_2 narcosis, first put forward to explain the Dilution Effect by Cohn (1918) and supported by Runnström, Tiselius & Lindvall (1945), could not be the correct interpretation in experiments in which CO_2 was continuously removed. Although there is little evidence to support the hypothesis of CO_2 narcosis, Rothschild's reasons for rejecting the hypothesis are not correct. It is true that the KOH in the centre well of a manometer flask can easily absorb all the CO_2 evolved in this type of experiment; but in fact, no 'CO_2 vacuum' is produced, but a steady state, in which, at any particular moment, there is far more 'CO_2' in a dense, than in a dilute, suspension. This significantly reduces the pH of the suspension (see, for example, Fig. 7).

There were two other observations concerning the Dilution Effect in this paper. First, that an atmosphere of pure O_2 has a more toxic effect on dilute than dense suspensions. This was not observed except when the dense suspensions exhibited symptoms of inadequate O_2 saturation. Secondly, that CO in the dark has a greater inhibitory action on dilute than on dense suspensions. The sperm densities in these experiments were 15, 7·5 and $3{\cdot}8 \times 10^7$/ml., so that questions of O_2 saturation should not have arisen. This experiment may show that there are physiological differences between dense and dilute suspensions, but no definite conclusions can be drawn until it is repeated using adequately buffered sea water.

(4) Rothschild & Tuft (1950) reported that the addition of $CuCl_2$ (5 p.p.m.) to dense suspensions of sea-urchin spermatozoa (*E. esculentus*) stimulated O_2 uptake, but that the same procedure inhibited the O_2 uptake of dilute suspensions. The pH of the medium was not taken into consideration and the experiments should therefore be repeated, under controlled conditions.

(5) Rothschild (1950) found that diethyldithiocarbamate inhibited the O_2 uptake of dense more than dilute suspensions of spermatozoa of *E. esculentus*. The pH was not controlled in these experiments. In this work and that referred to in (4), the question is raised as to whether the metabolic differences between dense and dilute suspensions are due to the amounts of copper in ordinary sea water being marginal when dense suspensions are under consideration.

(6) Tyler & Rothschild (1951) found that the Dilution Effect of the type shown in Fig. 1*b* could be abolished by adding sea water containing glycine, instead of sea water, to the sperm suspensions in the manometer flasks (*A. punctulata* and *Lytechinus pictus*). The experimental conditions precluded the possibility of the glycine effect being an artifact, though the experiment should be repeated with the pH of the medium controlled.

(7) On the basis of experiments in which sea water containing versene was found to inhibit the Dilution Effect (*E. esculentus*), Rothschild & Tyler (1954) put forward

the hypothesis that the respiratory Dilution Effect was caused by heavy metals in sea water. When present at a high enough concentration per spermatozoon it was suggested that they temporarily stimulate the respiration or O_2 uptake of sea-urchin spermatozoa, but also exert a toxic effect which is reflected in a reduction in viability or fertilizing life span. The implication of this hypothesis is that spermatozoa in dense and dilute suspensions are in different physiological conditions induced by trace metals in the external medium. Although no special precautions were taken to control pH in these experiments, it is difficult, if not impossible, to conceive that the extraordinary effects of versene in prolonging the functional life of sea-urchin spermatozoa could be reproduced simply by control of pH. If versene acts as a chelating agent and in other respects is biologically inert, the trace metal hypothesis is almost unavoidable, hard as it may be to believe that the quantities of copper or other heavy metals in sea water are so critical as to be relatively harmless to a suspension containing for example $7\cdot4 \times 10^8$/ml., but toxic to one containing half that sperm density.

MATERIAL AND METHODS

Semen of Echinus esculentus *Linn.* Differential manometers of the Warburg type were used in all experiments. The following details are self-explanatory: vol. 17–21 ml.; $kO_2 \doteqdot 1\cdot5$; 95 c./m.; 10% KOH and filter papers in centre cup; air in gas phase; T ° C. 15. O_2 saturation experimentally found to be adequate.

Sea water buffered with glycyl glycine (Tyler & Horowitz, 1937*b*), referred to as gly.gly.-sea water, was prepared by acidifying sea water containing 50 units penicillin/ml. to pH 3 with HCl, vigorously aerating for 24 hr. to drive off CO_2, adding the appropriate amount of glycyl glycine and adjusting the pH to the required value. At a concentration of 0·025 M, gly.gly.-sea water has no deleterious effect on sea-urchin spermatozoa.

RESULTS

pH and buffer capacity of sea water. The pH of outside filtered sea water at Millport varies between 7·90 and 8·20. In one experiment, 2 ml. of this sea water, pH 8·15, was placed in a manometer flask containing KOH and shaken for 75 min. At the end of the experiment, its pH had risen to 8·8. When sea water was put in the side-arms of a manometer flask, the increase during shaking was not so great, because the contents of side-arms are not agitated as efficiently as the fluid in the main part of the flask, and CO_2 removal is therefore less complete. In the above experiment, the pH of the sea water in the side-arms rose from 8·15 to 8·40. This may not seem a big change, but we shall see later that in such circumstances there may be a large pH difference between the fluid in the side-arms and the suspension in the main part of the flask at the time of mixing. The reason for the increase in the pH of sea water in a manometer flask containing KOH is, of course, the continuous removal of CO_2.

Apart from the undesirability of attempting manometric experiments involving the absorption of CO_2 when bicarbonate is present in a poorly buffered medium, the buffer capacity of sea water is low in comparison with the solutions normally used in metabolic experiments. Tyler & Horowitz (1937*b*) described some of the physico-chemical and biological properties of a bicarbonate-free sea water containing 25 μM/ml. glycyl glycine, and it is interesting to compare the buffer capacity of this solution with that of ordinary sea water (Fig. 2). The titration curve of the gly.gly.-sea water is similar to that published by Tyler & Horowitz. The comparable curve for ordinary sea water was obtained by adding different quantities of HCl to a series of vessels each containing 100 ml. of sea water, vigorously aerating them for 24 hr. and then measuring the pH of the acidified sea water in each vessel. That part of the curve to the right of pH 8·05 was obtained by adding NaOH to previously aerated sea water. Fig. 2 shows that both absolutely and relatively, sea water is a poor medium, from the point of view of buffer capacity, for manometric experiments in which CO_2 is continuously removed. This is not only because of the weak buffer

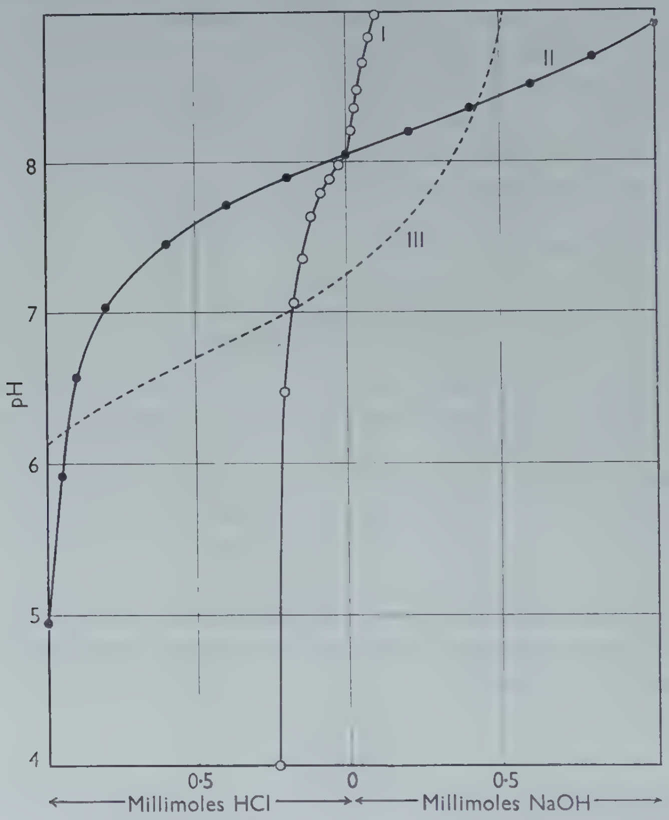

Fig. 2. Comparison of the buffer capacity of I, sea water; II, gly.gly.-sea water, 0·025 M; and III, mammalian sperm Ringer (Mann, 1954, pp. 77–8), used in manometric experiments in the region of pH 7.

capacity of sea water, but also because the process of experimentation interferes chemically with the buffer system, by turning bicarbonate into carbonate. It is, therefore, hardly to be expected that manometric experiments in which biological material is suspended in sea water and CO_2 is removed will be easy to interpret.

pH of sperm suspensions in sea water. Given these disadvantages in the use of sea water as a suspending medium, what actually happens to the pH of a suspension during an experiment? Fig. 3 shows the variation in the pH of a suspension containing $9{\cdot}76 \times 10^8$/ml. (semen diluted 1/25) for 30 min. after the addition of semen to sea water, without absorption of CO_2. The variations in the pH of the suspension at different times after the start of the manometric experiment, i.e. during absorption of CO_2, are shown in Fig. 4. Five manometers were used in this experiment, each containing 2 ml. of the 1/25 suspension and 0·2 ml. 10% KOH in the centre cup. It is not always easy to get five suspensions respiring almost exactly at the same rate, as in this case. During the course of the experiment the pH of the suspension changed from 7·45 to 8·16.

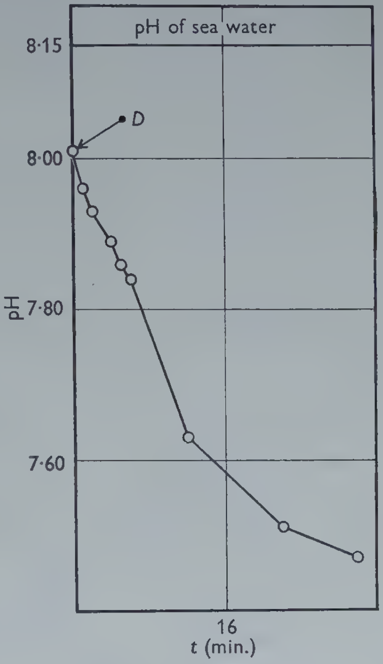

Fig. 3. Variation with time in the pH of a suspension of sea-urchin spermatozoa in sea water, $9{\cdot}76 \times 10^8$/ml., semen dilution 1/25, without absorption of CO_2. *D*, addition of semen to sea water.

Dependence of the Dilution Effect on the pH of the diluent. These experiments raised the suspicion that the Dilution Effect might be due, at any rate in part, to the diluent having a higher pH than the suspension to which it is added. An experiment to test this possibility is shown in Fig. 5. In each case the manometer flasks contained 1 ml. of a 1/25 suspension ($7{\cdot}28 \times 10^8$/ml.) of spermatozoa in sea water. At the beginning of the experiment, the pH of the suspension was 7·5. The side-arms contained 1 ml. of 0·05 M-gly.gly.-sea water, pH 8·3 in one case and pH 7·8 in the other. The experiment shows quite clearly that the Dilution Effect is negligible when the diluent has a pH of 7·8 but is very marked when the diluent has a pH of 8·3. At the end of the experiment, at $t = 130$ min., the pH of each suspension was 7·8 and 8·2. The very slight increase in O_2 uptake at 7·8 may have been due to there having been a difference between the pH of the diluent and that of the sperm suspension, the latter not having reached pH 7·8. The great sensitivity of sea-urchin sperm suspensions to the pH of the suspending medium is illustrated in Fig. 6, which shows the difference between the O_2 uptake of a suspension at about pH 8·0 and one at about 8·3. In this experiment the pH of the suspending medium, gly. gly.-sea water, 0·025 M, was adjusted in one case to 7·98 and in the other to 8·32.

After addition of semen (1/25) the pH fell to 7·84 in the first case and 8·22 in the second; but, at the end of the experiment, owing to the absorption of CO_2, the pH of each suspension had recovered to its original value. The sperm density was halved at $t = 80$ min. in both cases, but the pH of the diluent was the same as that of the suspension, and there was a negligible Dilution Effect.

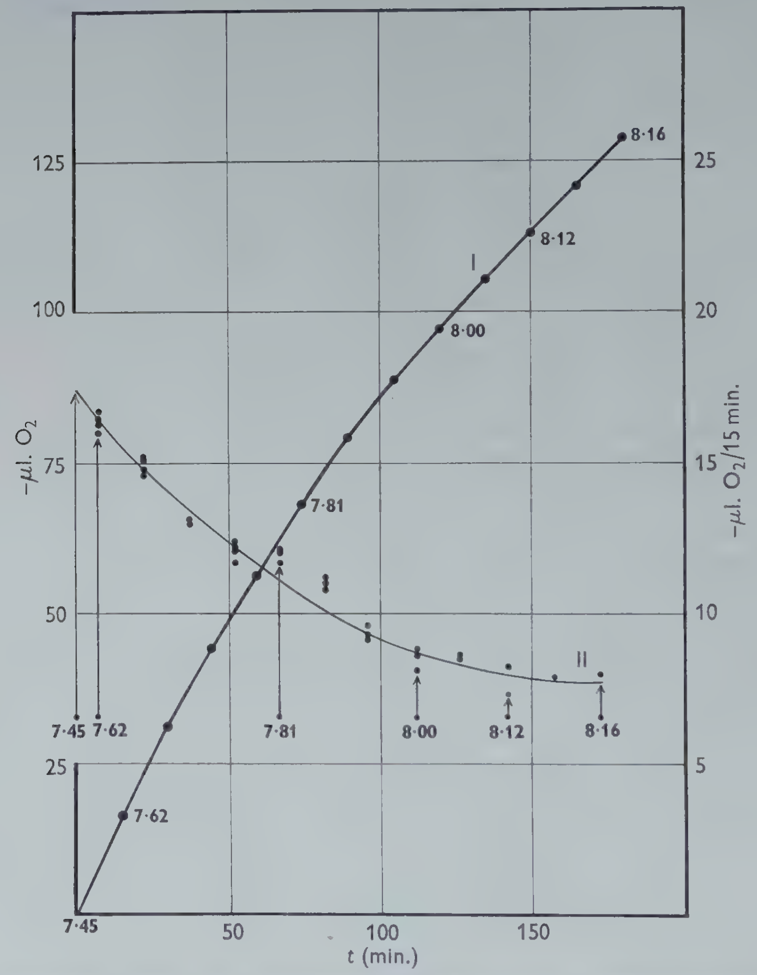

Fig. 4. Variation with time in the O_2 uptake and pH of a suspension of sea-urchin spermatozoa in sea water, density $9{\cdot}08 \times 10^8$/ml., semen diluted 1/25, with absorption of CO_2. The left-hand ordinates refer to the cumulative O_2 uptake curve, I; the right hand ordinates refer to the rate curve, II.

There is some evidence which suggests that the magnitude of the Dilution Effect is proportional to the difference between the pH of a suspension and the pH of the diluent which is added to it, provided that the diluent has a higher pH than the suspension. Table 1 summarizes an experiment which supports this thesis. Each flask (1–4) contained 1 ml. of a suspension containing $9{\cdot}52 \times 10^8$ spermatozoa in sea

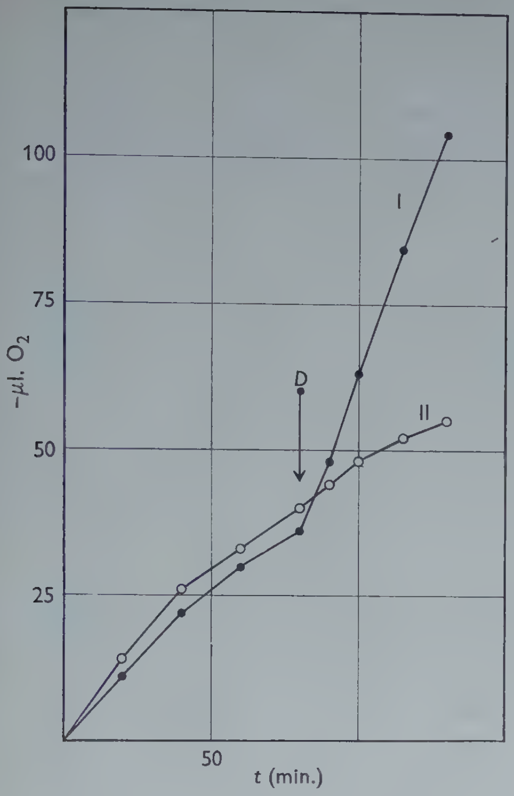

Fig. 5

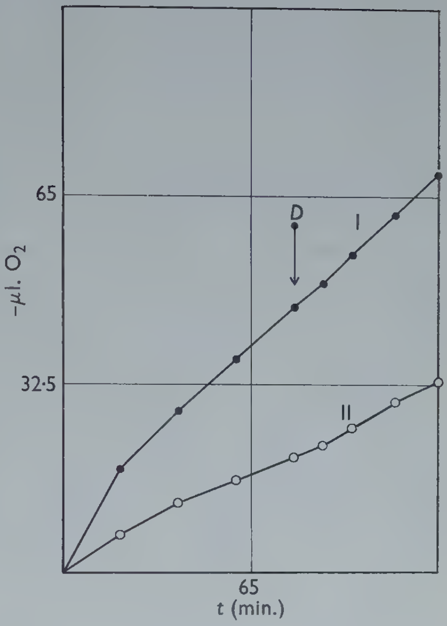

Fig. 6

Fig. 5. Effect of pH of diluent on O_2 uptake of sea-urchin spermatozoa. I, 1 ml. of a 1/25 suspension, containing $7{\cdot}28 \times 10^8$ spermatozoa, diluted 1/2 at *D* with 0·05 M gly.gly.-sea water, pH 8·3; II, identical with I, except that the pH of the diluent was 7·8.

Fig. 6. O_2 uptake of two suspensions of sea-urchin spermatozoa, per unit number of spermatozoa. I, $8{\cdot}44 \times 10^8$ spermatozoa in 1 ml. 0·025 M gly.gly.-sea water, pH 8·32. II, $9{\cdot}92 \times 10^8$ spermatozoa in 1 ml. 0·025 M gly.gly.-sea water, pH 7·98. At *D*, the sperm density was halved by addition from the side-arms of 1 ml. 0·025 M gly.gly.-sea water, pH 8·32 (I), and pH 7·98 (II). The difference between the number of spermatozoa in the two flasks was too small to cause any differential Dilution Effect.

Table 1. *Effect of pH of diluent on O_2 uptake of suspensions of sea-urchin spermatozoa in sea water*

Main vessel	1	2	3	4
	1 ml. sperm suspension, $9{\cdot}52 \times 10^8$/ml.			
Diluent	sea water, pH 8·2	gly.gly.-sea water, pH 8·3	gly.gly.-sea water, pH 8·0	gly.gly.-sea water, pH 7·5
r	1·50	2·86	1·00	0·33

r = (rate of O_2 uptake after addition of diluent)/(rate of O_2 uptake before addition of diluent).

water. The pH of the sea water was 7·90 and of the suspension, immediately after preparation, 7·29. After incubation for 80 min. the sperm density was halved by addition of 1 ml. of diluent from side-arms.

The experiments described above do not necessarily imply that sea-urchin spermatozoa in a dense sea water suspension, and therefore in a medium of low pH, are in the same physiological condition as those in a dilute sea water suspension, which are in a medium of higher pH. What has been shown is (1) that the O_2 uptake of suspensions containing the same number of spermatozoa increases with pH over a certain range, and (2) that the Dilution Effect is not observed when the pH of the diluent is about the same as that of the suspension to which it is added. This may not always hold good at a low pH, of the order of 7·6, because in an experiment described below and illustrated in Fig. 8, involving the addition of DNP to various suspensions, the rate of O_2 uptake of a dilute suspension (1/90, pH 7·66) was twice that of a dense suspension (1/30, pH 7·66). As the experiment was done for a different purpose, this point was not pursued; but if confirmed, it shows that although pH plays an important role in the Dilution Effect, other factors must also be taken into consideration, particularly at low pH's, as indeed is evident from a consideration of the effect of versene, discussed in the Introduction.

Effect of DNP on O_2 uptake and motility. Fig. 7 shows the results of an experiment to investigate this question, without special precautions to control pH. DNP in sea water, pH 8·1, was added from side-arms to three suspensions containing different numbers of spermatozoa in sea water. The DNP was added in a sufficiently small volume of sea water to obviate the possibility of there being any Dilution Effect. This was confirmed by simultaneously running control experiments in which the same amount of sea water, without DNP, was added to the suspensions. The following conclusions can be drawn from this experiment: first, that DNP stimulates the O_2 uptake of sea-urchin sperm suspensions; and secondly, that the stimulation is directly proportional to the sperm density. In Fig. 7, the ratio (rate of O_2 uptake after addition of DNP)/(rate of O_2 uptake before addition of DNP) is 1·14 for the most dilute suspension, 1·48 for the intermediate suspension, and 1·82 for the most dense suspension. The stimulation by DNP is also, however, inversely proportional to the pH of the suspension. At the end of the experiment depicted in Fig. 7, the most dilute suspension had a pH of 7·94, the intermediate one a pH of 7·65, and the most dense a pH of 7·51. The amounts of undissociated DNP in solution at these three pH's are $7{\cdot}2 \times 10^{-9}$ M, $1{\cdot}4 \times 10^{-8}$ M and $1{\cdot}9 \times 10^{-8}$ M.

DNP reduced sperm motility (visual assessment) in spite of stimulating O_2 uptake and, under these experimental conditions, without special pH control, the effect was more marked in dense than in dilute suspensions.

Fig. 8 shows the effect of controlling the pH of the suspending medium on the action of DNP. Although the stimulation of O_2 uptake is greater in the case of the dense suspension at pH 7·66, curve IV, than in the case of the dilute one at the same pH, curve III, the difference is not very marked. There is, however, a considerable difference between the stimulation of a dense suspension at pH 7·66, curve IV, and of a dilute suspension at pH 8·10, curve II. These two curves illustrate what would actually be observed if the suspending medium were ordinary sea water. One explanation of the difference between curves II and IV is the lower concentration of undissociated DNP at pH 8·10. Before considering the 'uncoupling' hypothesis as proved, it would be interesting to find out whether the permeability of a spermatozoon to undissociated DNP is independent of the pH of the suspending medium in the range pH 7·7–8·3. Some other possibilities, arising out of Mann's work on mammalian spermatozoa (1954), are considered in the Discussion section. Fig. 8 also illustrates a point which has been mentioned earlier; that at low controlled pH's there seems to be a definite physiological difference between dense and dilute spermatozoa, as the latter respire at a higher rate than the former.

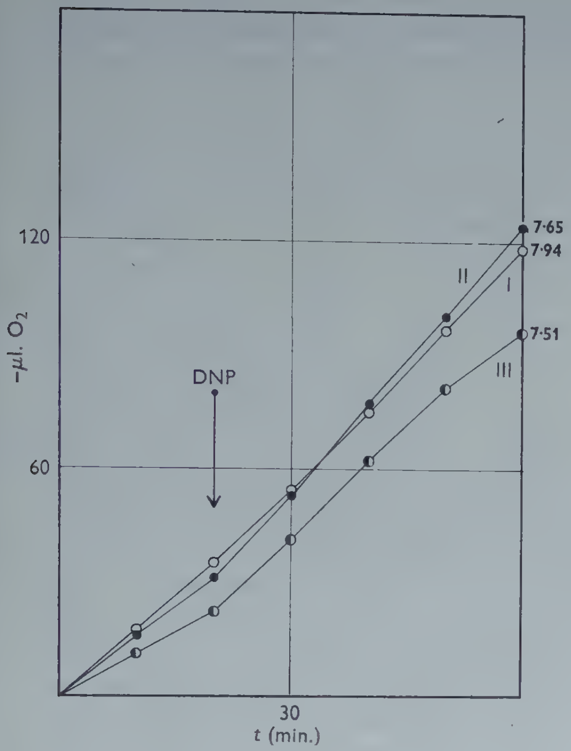

Fig. 7

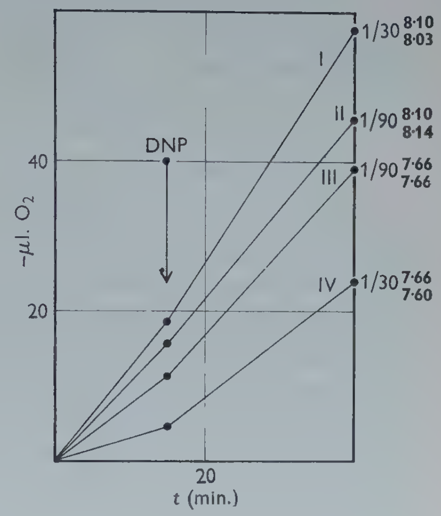

Fig. 8

Fig. 7. Effect of DNP, final concentration $6·3 \times 10^{-5}$ M, pH 8·1, on O_2 uptake, per unit cell number, of suspensions of sea-urchin spermatozoa in sea water. I, $3·25 \times 10^8$ in 2 ml.; II, $6·50 \times 10^8$ in 2 ml.; III, $1·30 \times 10^9$ in 2 ml. Note normal Dilution Effect between I and II, and III, before addition of DNP. The numbers on the right-hand side refer to the pH's of each suspension at the end of the experiment.

Fig. 8. Effect of DNP, final concentration $6·3 \times 10^{-5}$ M, pH 7·66 and 8·10, on the O_2 uptake, per unit cell number, of suspensions of sea-urchin spermatozoa in gly.gly.-sea water, pH 7·66 and 8·10. I, $5·00 \times 10^8$/ml., pH 8·10; II, $1·67 \times 10^8$/ml., pH 8·10; III, $1·67 \times 10^8$/ml., pH 7·66; IV, $5·00 \times 10^8$/ml., pH 7·66. Note lack of Dilution Effect at pH 8·10 and large Dilution Effect at pH 7·66. The numbers on the right-hand side refer to the pH's of each suspension at the beginning and end of the experiment.

Antagonism between versene and DNP. The trace metal explanation of the Dilution Effect and its inhibition by versene were mentioned in the Introduction; it was, therefore, natural to enquire whether the stimulation of O_2 uptake and the impairment of motility induced by DNP could be counteracted by versene. If sea-urchin sperm suspensions are made up in sea water containing 10^{-3} M versene, pH 8·0, the impairment of motility observed after addition of DNP is strikingly reversed. The stimulation of O_2 uptake by DNP is also reversed, to a certain extent, by versene (Table 2).

Table 2. *Effect of DNP, final concentration* $6{\cdot}28 \times 10^{-5}$ M, *pH* 8·0, *on the* O_2 *uptake of sea-urchin sperm suspensions, at different sperm densities, with and without versene,* 10^{-3} M, *pH* 8·0, *in the suspending medium*

Sperm density	$7{\cdot}28 \times 10^8$/ml.		$3{\cdot}64 \times 10^8$/ml.		$1{\cdot}82 \times 10^8$/ml.	
Suspending medium	s.w.*	v.s.w.†	s.w.	v.s.w.	s.w.	v.s.w.
r	1·80	1·33	1·46	1·20	1·11	1·20

* s.w., sea water. † v.s.w., sea water containing versene.
r = (rate of O_2 uptake after addition of DNP)/(rate of O_2 uptake before addition of DNP).

The ratios in the s.w. columns show that, as in the previous set of experiments, the stimulating action of DNP is directly proportional to sperm density (and inversely to the pH of the suspension). In spite of these experiments being done with ordinary sea water as the suspending medium, their results support the hypothesis that both DNP and trace metals in ordinary sea water may act on the same enzyme systems within sea-urchin spermatozoa.

Effect of usnic acid on O_2 *uptake and motility.* I have been unable to confirm the results reported by Nelson (1948) which were mentioned in the Introduction; but as few experimental details are given in his brief note, the conditions in these experiments may not have been the same as in his. The usnic acid was suspended in sea water or gly.gly.-sea water containing 0·01 % gelatin, the amount suspended being approx. 100 μg./ml. There is no information as to how much goes into solution in such circumstances, but to conform so far as possible with Nelson's experiments, suspensions and not solutions of usnic acid in sea water were used. Usnic acid is toxic to these sea-urchin spermatozoa, the toxicity being more marked in dilute than in dense suspensions. In an experiment in which slightly diluted semen (1/2·5) was added from a side-arm to sea water containing different concentrations of usnic acid (140, 70 and 35 μg./ml.) in the main compartment, the spermatozoa were about one-third as active as the controls, after 60 min. incubation. The final semen dilution was 1/27·5. When the same experiment was done in such a way that the final semen dilution was 1/82·5, all the spermatozoa in the suspensions containing usnic acid were dead after 60 min. In this experiment the pH was controlled by using 0·025 M gly.gly.-sea water containing 0·01 % gelatin, pH 8, as the suspending medium throughout. This experiment supports the view that there are physiological differences between dense and dilute suspensions of sea-urchin spermatozoa. Although there was little evidence of any significant stimulation of O_2 uptake, something unexpected was observed both in dense and dilute suspensions. When slightly diluted semen is washed from a side-arm into the main compartment, the O_2 uptake of the suspension is often higher in the second reading period (e.g. 15–30 min.) than in the first (0–15 min.). When the spermatozoa are known or believed to be ripe, this is probably due to inadequate mixing initially, or, perhaps, to a delay in the spermatozoa becoming fully activated, though the latter is sometimes said to be a characteristic of under-ripeness. If the diluent contains usnic acid, this delay is not observed and, at the beginning of the experiment, the O_2 uptake of the usnate-containing suspension is much greater than that of the controls. The effect is transient (see Fig. 9) where it can be seen to occur in a dense (1/27·5) and a dilute (1/82·5) suspension. The same phenomenon was observed when the experiment was done without pH control. This method of diluting semen or slightly diluted semen is unsatisfactory when readings are required a few minutes later, because of the difficulty of quickly mixing the viscous contents of the side-arm with the diluent in the main compartment. When the experiment was done the other way round, i.e. by adding the diluent from side-arms to the sperm suspension in the main

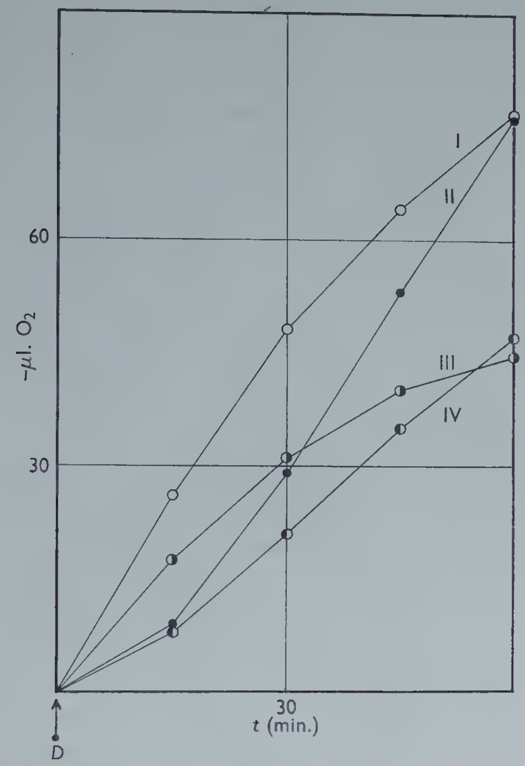

Fig. 9. Effect of usnic acid, 140μg./ml., on the O_2 uptake, per unit cell number, of suspensions of sea-urchin spermatozoa in gly.gly.-sea water containing 0·01 % gelatin, pH 8·0. I, $8{\cdot}87 \times 10^8$/ml. with usnic acid; II, $8{\cdot}87 \times 10^8$/ml. without usnic acid; III, $2{\cdot}96 \times 10^8$/ml. with usnic acid; IV, $2{\cdot}96 \times 10^8$/ml. without usnic acid.

compartment, only a small increase in O_2 uptake was observed on addition of a diluent containing usnic acid; the increase was a little more marked at pH 8·0 than at pH 7·7.

DISCUSSION

The following conclusions can be drawn from the experiments described in this paper. First, that because of the sensitivity of sea-urchin spermatozoa to changes in the pH of the medium, manometric experiments involving the absorption of CO_2 should not be done with ordinary sea water as the suspending medium. The results of such experiments cannot be interpreted because of the large pH changes which occur under these conditions. The respiratory Dilution Effects previously observed after addition of sea water to dense suspensions, whether the dilution was made before or during the manometric experiment, may therefore have been 'artifacts', due to the increased sperm activity at high pH's.

Except at rather low pH's, of the order of 7·65 (see Fig. 8), it is not certain that the classical respiratory Dilution Effect occurs. At the same time, there is no doubt that sea-urchin spermatozoa in a dilute suspension die more quickly than in a dense suspension, though the pH dependence of this characteristic property has not been adequately investigated. Assuming, however, that this property is real, there is an *a priori* case for the existence of a physiological difference between sea-urchin spermatozoa in dense and dilute suspensions, even in a medium of constant pH. This difference resembles that between dense and dilute mammalian sperm suspensions; but as the addition of versene to sea water does not stabilize its pH, the loss of sperm viability in a dilute suspension is almost certainly associated with the ratio (trace metal/ml. sea water)/(spermatozoa/ml. sea water). Although versene strongly chelates calcium, Tyler (1953) concluded, on the basis of experiments in which the concentration of calcium in an artificial sea water was varied over wide limits, that calcium could not be responsible for the Dilution Effect. This view was subsequently reaffirmed (Rothschild & Tyler, 1954). Tyler showed that a dilute suspension was not helped by reducing the calcium content of the suspending medium and that, both in dense and dilute suspensions, the life-span of sea-urchin spermatozoa was maximal at a calcium concentration of 0·01 M, which is about the value in normal sea water. Tyler's figures show that an abnormally high calcium concentration is invariably harmful; they also leave no doubt that a reduction in calcium concentration is not beneficial to dense suspensions and is positively harmful to dilute suspensions. Vasseur's experiments (1949), in which the addition of isotonic $CaCl_2$ solutions to sea-urchin sperm suspensions caused a large increase in O_2 uptake, should be repeated with pH control of the suspending medium. In any case, the experiments were done with semen diluted 1/4·5 and 1/6 with sea water, in which conditions there was no possibility of adequate O_2 saturation in the conical manometer flasks he used.

The second conclusion to be drawn from the experiments described in this paper is that a case can be made, though it is not proved, that the Dilution Effect is caused by the uncoupling of oxidation and phosphorylation and that trace metals in sea water are responsible for this phenomenon. The direct evidence in support of this view is first, that when pH is controlled, DNP stimulates the O_2 uptake of dense suspensions more than dilute ones, though the effect is not so marked under these conditions as when normal sea water is the suspending medium. Secondly, that in the presence of versene, the effect of DNP is reduced. The first piece of evidence suggests that there is a difference between sea-urchin spermatozoa in dense and dilute suspensions and that this difference is connected with the P/O ratios appropriate to the different types of suspension. As regards the protection afforded by versene, Abood & Romanchek (1955) showed that the inhibition of phosphorylation in rat-brain mitochondria induced by electrical stimulation could be almost completely reversed by 10^{-3} M versene. If, as these workers imply, DNP and electrical stimulation act, directly or indirectly, on the same enzyme systems, in the sense that both uncouple oxidation and phosphorylation, one might predict from their experiments that versene would reverse the inhibition of phosphorylation induced by DNP. Versene certainly reduces the effect of DNP on sea-urchin spermatozoa.

Abood (1954) reported in an earlier paper that the inhibition of oxidative phosphorylation by electrical stimulation varied with the composition of the electrodes; but in the paper referred to above, he and Romanchek suggest that this variability may have been due to the presence of very small quantities of heavy metals or electrolytically produced oxidizing radicals, such as OH or H_2O_2, catalysing the oxidation of thiol groups—also achieved by electrical stimulation. Quite apart from the experiments on sea-urchin spermatozoa, this raises the possibility that DNP and heavy metals may in some circumstances act in a similar way and inhibit oxidative phosphorylation.

The ability of versene to protect rat-brain mitochondria against the effects of electrical stimulation and to inhibit the action of DNP on sea-urchin spermatozoa suggests that the inhibition of oxidative phosphorylation requires the presence of some heavy metal. But when ferricyanide was substituted for oxygen as an electron acceptor, Abood & Romanchek found that versene did not inhibit the inhibition of phosphorylation by electrical stimulation. Although this confirms that electrical stimulation *per se* is responsible for the inhibition, it is not obvious why versene should protect mitochondria in the presence of oxygen but not when it is replaced by ferricyanide. According to these workers (1955, p. 233), 'independently of any effect on heavy metals, glutathione and certain other chelating and reducing substances will protect against the inhibition of phosphorylation by electrical currents'.

Apart from the apparent antagonism between versene and DNP, the observations recorded here and in Abood & Romanchek's paper are consistent with the following scheme for oxidative phosphorylation:

$$(1)\quad AH_2+B+C \rightleftharpoons A\sim C+BH_2,$$

$$(2)\quad A\sim C+ADP+H_3PO_4 \rightleftharpoons A+C+\mathrm{ATP},$$

$$(3)\quad A\sim C \rightarrow A+C.$$

AH_2 and B are adjacent members of the respiratory chain, C being a substance required for their interaction, though it does not undergo oxido-reduction. Reactions (1) and (2) occur in intact mitochondria, but (3) only comes into operation when the mitochondria are damaged, or in the presence of DNP. Reaction (1) can proceed in the forward direction, i.e. there will be respiration, only when C is regenerated from $A \sim C$. In intact mitochondria this regeneration requires ADP and H_3PO_4. When one or other of these is used up, respiration ceases. It will, however, be promoted by any agent, such as DNP, which brings about reaction (3) or promotes the hydrolysis of ATP.

A heavy metal may act like DNP in the sense of increasing the ATP-ase activity of the system. If the heavy metal promotes reaction (3), 'ATP-ase' is the backward reaction (2) + reaction (3). Alternatively, the heavy metal may increase the activity of a separate ATP-ase. The increase in the O_2 uptake of sea-urchin spermatozoa in the presence of heavy metals can be interpreted along these lines, but as the increase is not geared to the synthesis of ATP, the energy-rich bond being lost by reaction (3), there will be a loss of viability.

SUMMARY

1. Sea-urchin spermatozoa (*Echinus esculentus*) are extremely sensitive to changes in the pH of the suspending medium, their respiration being proportional to pH between 7·6 and 8·4.

2. In a manometric experiment in which semen was diluted 1/25 with sea water (CO_2 absorbed), the pH of the suspension was 7·5 at the beginning of the experiment and 8·2 at the end, after 180 min. incubation at 15° C.

3. Sea water, buffered with glycyl glycine, 0·025 M, and brought to pH 8·3, was added to a sperm suspension whose pH was 7·5 at the beginning of the experiment, after a period of incubation. There was a pronounced respiratory Dilution Effect. When the sea water was buffered with glycyl glycine and brought to pH 7·8, and this diluent was added to the same sperm suspension, there was a negligible respiratory Dilution Effect.

4. The O_2 uptake of suspensions prepared in buffered sea water at pH 8·3 was markedly higher than that in the same buffered sea water at pH 8·0.

5. These observations cast doubt on the reality of the respiratory Dilution Effect when observed in experiments in which sea water is the suspending medium and respiration is measured by a method involving the absorption of CO_2. An exception to this generalization was observed in buffered sea water at a low pH, 7·6. In this case, the O_2 uptake of dilute suspensions was greater, per unit number of spermatozoa, than that of dense suspensions.

6. 2,4-dinitrophenol, 6×10^{-5} M, stimulated the O_2 uptake and depressed the motility of spermatozoa more in dense than in dilute suspensions. Versene, 10^{-3} M, reversed the action of 2,4-dinitrophenol.

7. The results of other workers on adding suspensions of usnic acid to sea-urchin spermatozoa were not confirmed. This substance is more toxic to spermatozoa in dilute than in dense suspensions.

I am indebted to the Medical Research Council for the provision of a laboratory assistant.

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ENDOCRINE ACTIVITY DURING INSECT EMBRYOGENESIS. FUNCTION OF THE VENTRAL HEAD GLANDS IN LOCUST EMBRYOS (*LOCUSTANA PARDALINA* AND *LOCUSTA MIGRATORIA*, ORTHOPTERA)

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(With Plate 3)

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INTRODUCTION

The subject of insect endocrinology has been largely concerned up till now with the problem of metamorphosis. Up to the present the outcome of this has been to show that there exists in insects a tripartite endocrine system composed of the brain, corpora allata and the thoracic glands (ventral glands, pericardial glands, lateral cells or peritracheal glands of Weismann). Fukuda (1951, 1952) has also demonstrated the source of a diapause hormone, in *Bombyx*, as being located in the suboesophageal ganglion.

The problem of the relationship of the brain to the thoracic glands was finally resolved by Williams (1952). The evidence obtained from his studies on the *Cecropia* silkworm clearly showed that a brain factor liberated by the neurosecretory cells of the pars intercerebralis induces the thoracic glands to secrete their contributive hormone.

There is also abundant proof, furnished particularly by Wigglesworth (1948, 1952, *Rhodnius*), that the hormone released by the corpora allata, when present at a required level of concentration before a certain critical period during the growth of a larva or nymph, prevents progress in differentiation and growth towards the adult.

However, it was apparent that there was much that remained obscure concerning the relation of the thoracic glands to the moulting process, differentiation and growth in the adult direction and the termination of diapause. Moreover, the prospect of resolving this central problem by further work on the metamorphic phase did not appear to be particularly encouraging.

Thus, attention was directed towards the embryonic phase, and the chief aim of the present investigation has been an attempt (*a*) to discover the precise function of the thoracic glands (ventral head glands), and (*b*) to explain the initiation of post-diapause development in young embryos.

MATERIAL

This communication is based on a study of the diapausing eggs of *Locustana pardalina*, the South African brown locust, and the non-diapausing eggs of *Locusta migratoria*, the migratory locust.

In the natural environment the embryo of *pardalina* at a certain stage in its development enters diapause which lasts for about 6 months. At the end of diapause development will recommence, provided there is sufficient moisture. If, on the other hand, dry conditions prevail on the termination of diapause further progress in development will be delayed. Dried eggs in this quiescent state are capable of remaining viable for several years. Matthee (1951), who very kindly made the dried eggs of *pardalina* available for this work, has pointed out these features.

As far as the present work was concerned, it was of value to know, first, that dried eggs over 6 months old would recommence development on wetting. Secondly, that the embryo was invariably placed in a set position, namely, the diapause position, when development recommences.

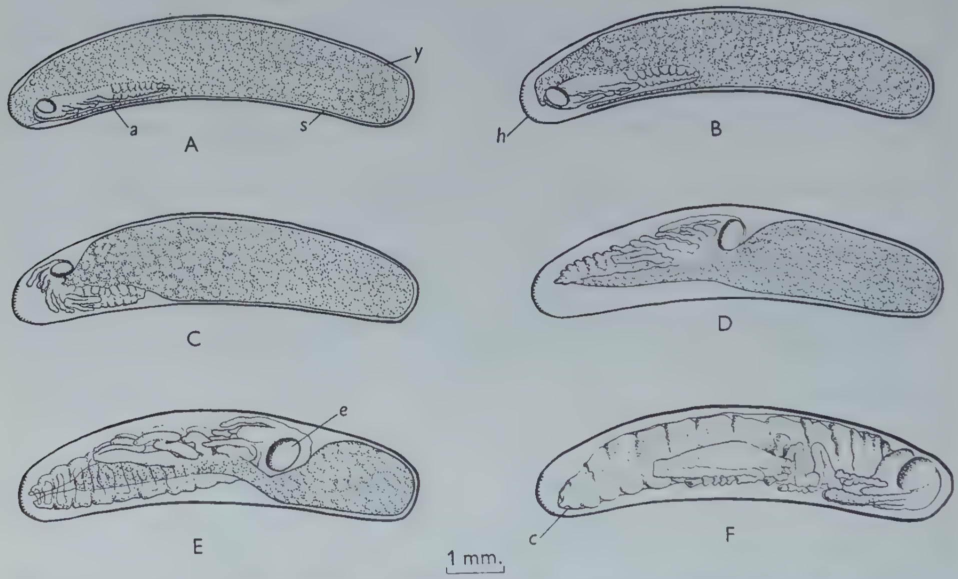
Males and females of *migratoria*, generously provided by the Anti-locust Research Centre in London, were kept in cages and freshly laid pods of eggs were obtained from fertile females.

STAGES IN EMBRYONIC DEVELOPMENT

Efforts were first directed towards tracing and timing significant events in embryogenesis. The locust egg when freshly laid largely consists of yolk surrounded by a blastoderm enclosed within an outer chorion.

The embryonic phase of development in *migratoria* lasted about 14–15 days at 30° C., 100 % R.H. Within 3 days the extra-embryonic serosal layer, derived from the blastoderm and enclosing the yolk, had completed its deposition of the serosal cuticle and the formation of the hydropyle at the posterior pole. On the 5th day the embryo, already resembling the prospective locust, had taken up a position on the ventral side of the egg with its head adjacent to, and pointing towards, the hydropyle and its appendages facing the chorion (Text-fig. 1 A). The embryo, except for the dorsal surface, was now surrounded by the amniotic cavity. The amnion itself below the head was fused with the serosa, and it was here that the embryo broke through prior to revolving around the posterior pole, a process, namely, katatrepsis, which took place and was completed during the 7th day (Text-fig. 1 B, C, D). By the end of the next day the embryo had grown to the length of the egg, having meanwhile rolled 180° around the longitudinal axis of the egg to bring its dorsal side against the dorsal surface of the egg. On this 8th day a moult occurred, the epidermis retracting away from a provisionally laid cuticle.

The prospective gut became enclosed on the 7th day, but it retained its continuity with the serosa sac at the cervical region immediately behind the top of the head. On the 7th and 8th days the embryo grew rapidly. The prospective gut increased in size and became filled with yolk transferred from the serosal sac which compensatingly decreased in size. On the embryo reaching the full length of the egg, all the available yolk was contained in the gut; a cervical pouch remained to indicate the previous existence of the serosal sac. Thereafter, further changes were directed towards the development of the features to be seen in the locust on the 14th–18th day when the egg hatched. The stages from the 5th to the 9th day are illustrated in Text-fig. 1.



Text-fig. 1. Embryonic development in a locust, based on studies of *Locustana pardalina* and *Locusta migratoria*. A, embryo upon the ventral side of the egg (the diapause position in *L. pardalina*); B, amnio-serosal membranes have ruptured; C, embryo revolving; D, katatrepsis completed; E, body has fused in the mid-dorsal line to enclose the prospective gut; gut still connected through dorsal pore with yolk contained in the serosa lsac; F, lateral rotation has taken place and the embryo has attained the length of the egg; dorsal pore has closed; and the provisional cuticle has been shed. *a*, amnion; *s*, serosa; *y*, yolk; *h*, hydropyle; *e*, presumptive eye-disk; *c*, shed cuticle.

As already mentioned *pardalina*, unlike *migratoria*, enters a diapause phase during embryogenesis. Prior to this the embryo reaches a stage in development corresponding to that reached by the embryo of *migratoria* on the 5th day (Text-fig. 1 A).

The position in which the embryo is orientated at this time is retained throughout the diapause period. Exposure of the egg to moisture during diapause, only results in an uptake of water and a very slight enlargement of the embryo (Matthee, 1951).

However, exposure of dried quiescent eggs to moisture invariably induced a recommencement of development. The following observations were made on eggs kept moist at 30° C. In 2 days the eggs were turgid (Text-fig. 1 A). On the 3rd and 4th days the embryo increased its size. During the 5th day, the embryo grew rapidly, completed katatrepsis and reached the customary position on the dorsal side of the egg. At the end of this 5th day the embryo had attained a length about half that of the egg (Text-fig. 1 D).

The rapid rate of growth was continued, being expressed the next day by the embryo increasing its length to that of the egg. On this 6th day there was a moult, the epidermis retracting from the provisional cuticle (Text-fig. 1 F).

Further progress up to the time of hatching was in some respects an anti-climax. Prior to hatching the chorion persisted as fragmentary strips attached to the serosal cuticle. The inner part of this cuticle was dissolved away leaving a thin outer layer (the 'yellow' layer as opposed to the inner 'white' one), which gave way as the locust, still enveloped in its provisional cuticle, forced its way out at the anterior end. The provisional cuticle was discarded soon after emergence.

In view of the aims of the present investigation there emerged from these observations, particularly in the case of *pardalina*, two significant processes. They were (*a*) the initiation of growth consequent upon exposure to moisture and (*b*) the moult of the provisional cuticle midway through embryogenesis.

CHANGES IN THE ENDOCRINE CENTRES PRESENT IN THE EMBRYOS

The immediate aim arising from the foregoing observations on the stages of embryonic development in both *migratoria* and *pardalina* was to try to discover whether endocrine centres, of the kind known to exist in post-embryonic stages, operated during embryogenesis, and if they were present and active to determine the relation of the centres to each other, to the moult and, particularly in the case of *pardalina*, to the initiation of growth and differentiation following the termination of diapause.

Serial sections of embryos of *migratoria* and *pardalina** in different stages of development were therefore prepared and treated with Gomori's chrome-haematoxylin phloxin, a stain which shows up neurosecretory cells very clearly.

The corpora allata in *pardalina* and *migratoria* had assumed their customary position on either side of the oesophagus and also their characteristic oval shape on the completion of katatrepsis.

The presence of a pair of ventral head glands, homologous with the thoracic glands of other insects, was first detected in *pardalina* with certainty in sections of embryos which had completed katatrepsis (Jones, 1953). These glands were also located in *migratoria*, and Strich (1954) has described them in the post-embryonic stages of this locust.

The glandular character of these organs situated in the posterior region of the head was most readily displayed in sections of embryos which had attained a length about equal to that of the egg. In *migratoria* these organs were elongated, tapering towards each end and widest across the middle (Text-fig. 2). One end originated in the epidermis from a dorso-lateral position posterior to the system of dorsally situated head muscles, the gland curving inward and extending downward to rejoin the epidermis at a point opposite the sub-oesophageal ganglion. The cytoplasm was syncytial and contained deeply stained nuclei of varying shapes and sizes. Some of the nuclei were lobulated. In *pardalina* the ventral head glands at a corresponding stage in development differed in some respects from those in *migratoria*. For instance, in *pardalina* these organs did not appear to taper at each end before condensing with the epidermis.

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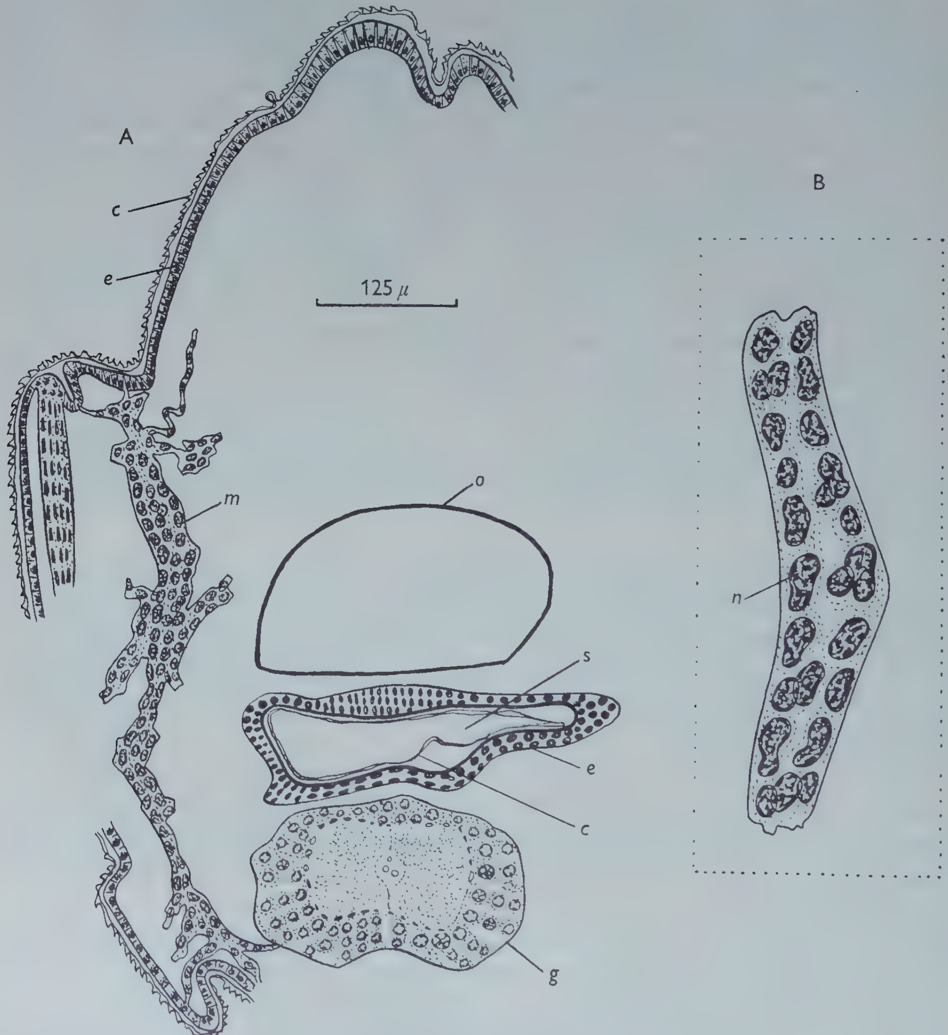
* I wish to acknowledge the help of Dr Wallace and Mr Sharan in producing Pl. 3.

The presence of neurosecretory cells was first established in *pardalina*. They were placed at the periphery of the protocerebrum. When the quiescent eggs had



Text-fig. 2. Appearance of a moulting gland in the embryo of *Locusta migratoria* immediately after the retraction of the epidermis from the cuticle. A, moulting gland reconstructed from serial sections; it extends backward and downward throughout 14 sections of 8 μ thickness; B, section of part of the gland. *m*, moulting gland; *e*, epidermis; *c*, shed cuticle; *o*, area occupied by the oesophagus in the top section; *g*, sub-oesophageal ganglion as it appeared in the last section of the series; *n*, nucleus; *s*, space bounded by the epidermis in the last section of the series.

become turgid on the 2nd day after being wetted, some nerve cells were seen to be larger than the others. On the 3rd day, in the same region of the protocerebrum, there were to be seen relatively large cells which had developed the histological appearance of neurosecretory cells. The nuclei of these cells were indistinguishable from the normal nerve-cell nuclei, but, on the other hand, the cytoplasm contained material which stained blue with chrome-haematoxylin.

However, on the 4th day these cells showed all the histological signs of being neurosecretory cells. For instance, by this time they had also acquired large vacuoles. It was interesting that by the end of the following day it was most difficult to distinguish neurosecretory cells from the other nerve cells, in particular, the neuroblasts. Thus, judging by the evidence obtained from an examination of serial sections, it appeared as if the neurosecretory cells had reached their period of maximal activity on the 4th day (Pl. 3 B).

On this 4th day after the quiescent eggs had been wetted, there was no sign of the ventral head glands. However, on the 5th day it was possible to locate groups of cells in those positions where one would expect the glands to be developing. It was remarkable that on the next day the glands were differentiated and had also reached their maximal phase of activity and peak size. The bulk of the cytoplasm had increased and so had the numbers of nuclei which varied in both shape and size (Pl. 3 C). By the next day the glands had become reduced in size. By the 9th day they were less than half their maximal size, and they remained in this condition up to the time of hatching which occurred a few days later (Pl. 3 D).

Of considerable significance was the coincidence of this maximal period of activity of the ventral head glands with the retraction of the epidermis from the cuticle, an event which, as already mentioned, also occurred on the 6th day. This synchrony between these two events was also seen in *migratoria*.

The corpora allata, in both *pardalina* and *migratoria*, showed no visible change either in size or in histology, throughout embryonic development, after they had become fully differentiated.

In view of these observations, indicating the periods during post-quiescent development when the endocrine tissues became organized and functional after wetting the quiescent egg of *pardalina*, the next step was to ascertain at what stage the cells first showed visible signs of actively dividing. The serial sections of embryos furnished this information. In sections of embryos removed from the egg on the 3rd day after wetting, or the day after the egg had become fully turgid, mitotic figures were clearly displayed in the cells, in particular, those of the nervous system and the foregut. No such evidence of cell division was to be seen in sections of embryos removed before the end of the 2nd day when the eggs became fully turgid. From the 3rd day mitosis continued up to the 8th day, that is to say, about 3 days before hatching.

This renewed burst of mitosis, therefore, began in *pardalina* at least 3 days before either the corpora allata or the ventral head glands had been formed and at least 4 days before the latter organs had reached their maximal phase of activity.

LIGATURING EXPERIMENTS ON ISOLATED EMBRYOS

The evidence presented so far indicates that there was no participation of organized and functional endocrine tissue in the initiation of post-quiescent (post-diapause) growth in the egg of *pardalina*.

Efforts were next made to ascertain the effects of performing ligaturing experiments on isolated embryos designed to discover just what relationship existed between the ventral head glands and the moult, which appeared to entail nothing more than the retraction of the epidermis from the cuticle; for in the embryo there was no need for any special activation of the epidermis just before the moult because the epidermis had already been activated 3 days previously, and 2 days before the ventral head glands were formed.

Embryos were ligatured either between the head and thorax, or between the thorax and abdomen, so that in each case the embryo was divided into two blood-tight compartments. Experiments were performed on embryos which had attained either about two-thirds or about three-quarters of the length of the egg. Notwithstanding the opacity of the chorion it was not difficult to make out the position of the embryo. However, if necessary, the chorion could be readily removed by immersing the eggs in bleach solution, a treatment which did not impair the viability of the contained embryo.

The eggs were placed in a Petri dish containing sterile Ringer's solution (0·75 or 0·9 % NaCl, 0·02 % KCl, 0·02 % $CaCl_2$ and 0·02 % $NaHCO_3$). The anterior end of the egg was first punctured to release the internal pressure. Some of the yolk escaped when this was done. The collapsed end was cut off and the embryo was gently pressed out through the opening into the solution. The extra-embryonic serosa was invariably torn when this operation was carried out.

The yolk in the remaining part of the serosal sac was removed before trimming the open end so as to enable the serosa to condense more easily into a pad of tissue closing off the prospective gut and its supply of yolk from the outside medium. It usually took about 10 min. for the serosa to form this pad. When this was accomplished properly it was noticeable that the embryo was able the more successfully to withstand the ensuing ligaturing operations.

Embryos which came through the ligaturing operation with no injury, beyond that of being divided into two compartments, displayed strong pulsating movements along the heart region. When the ligature was applied between the thorax and abdomen the heart in both compartments continued to beat. The display or cessation of these heart movements served as a useful indication of the state of the experimental embryos. The isolated embryos, whether they were ligatured or used as controls, were placed in glass cells containing aerated sterile Ringer's solution at 20° C. Reasonable sterile precautions were taken because otherwise the isolated embryos deteriorated within a few days. Glassware and other materials were exposed to ultra-violet (wave-length 2537 A.) before use and the usual precautions were taken over needles and pipettes. Streptomycin sulphate and/or either potassium or sodium penicillin G were added to the Ringer's solution to eliminate bacterial infection.

The self-sustaining embryos remained alive in aerated sterile Ringer's for about 2 weeks, and during this time they showed signs of progress in development, for example, the spreading of the pigmentation zone across the presumptive eye disk and the moulting of the provisional cuticle. Slifer (1934) maintained that isolated embryos of the grasshopper, *Melanoplus differentialis*, cultured *in vitro*, acquired the characteristics of a locust about to hatch out of the egg.

In the case of *migratoria*, viable preparations of embryos about two-thirds the length of the egg moulted within 4 days. In six out of sixteen preparations of this stage, ligatured between the thorax and abdomen, the moult was limited to the thorax. The remaining preparations either failed to moult within 4 days, or deteriorated too rapidly to produce any result (Table 1). In six out of ten viable preparations ligatured between the head and thorax the body failed to moult (Table 1). Of the controls five in eight preparations moulted in about 4 days.

Table 1. *Effect of ligaturing post-katatrepsis embryos of* Locusta migratoria *which had attained a length of two-thirds that of the egg*

Ligatured between the thorax and abdomen			Ligatured between the head and thorax			Controls		
No.	Moult limited to thorax	Failed to moult within 4 days	No.	Body failed to moult within 6 days	Deteriorated within 4 days	No.	Moulted within 4 days	Dead within 4 days
16	6	10	10	6	4	8	5	3

Table 2. *Effect of ligaturing post-katatrepsis embryos of* Locusta migratoria *which had attained a length of three-quarters that of the egg*

Ligatured between the thorax and the abdomen				Controls	
No.	Moult limited to thorax	Thorax and abdomen moulted	Failed to moult within 4 days	No.	Moulted within 4 days
16	3	6	7	6	6

Preparations of embryos of *migratoria* which had attained a length about three-quarters that of the egg also moulted within 4 days. Of a total of sixteen preparations ligatured between the thorax and abdomen, the moult in three cases was limited to the head and thorax, and in six cases the moult occurred in both the thorax and the abdomen (Table 2). The remainder either failed to moult within 4 days, or deteriorated too rapidly to produce a result. Of the controls those that remained viable for long enough moulted.

The evidence obtained from these tests on *migratoria* indicated that the 'critical' period for the moult coincided with the embryo reaching a length of approximately three-quarters that of the egg.

In the case of *pardalina*, only embryos about two-thirds the length of the egg were tested. This length was achieved on the fifth day after wetting the quiescent egg.

Of those ligatured between the thorax and abdomen, the moult in four cases out of twenty, two out of nine, six out of seventeen and two out of fourteen was limited to the thorax (Table 3). Of a total of eight ligatured between the head and thorax, the body in all of them failed to moult (Table 3). Of the control preparations all of them moulted.

The first signs of a moult having taken place in the thorax of ligatured embryos were best seen by examining the tips of the legs, for the embryonic cuticle, if shed, appeared as a thin membrane stretched tightly over the claws. When the moult occurred in the abdomen the shed cuticle became stretched over the projections at the extremity.

The results of these experiments indicated that the source of the moulting factor was located in the head region, thus supporting the view gained from histological studies that the ventral head glands were directly implicated in the moult.

Table 3. *Effect of ligaturing post-katatrepsis embryos of* Locustana pardalina *which had attained a length of two-thirds that of the egg*

Ligatured between the thorax and abdomen			Ligatured between the head and thorax		
No.	Moult limited to thorax	Either failed to moult or deteriorated within 4 days	No.	Failed to moult within 4 days	Deteriorated within 4 days
20	4	16	16	12	4
9	2	7	—	—	—
17	6	11	—	—	—
14	2	12	—	—	—
2	1	1	—	—	—

Controls: six of eight preparations moulted within 4 days.

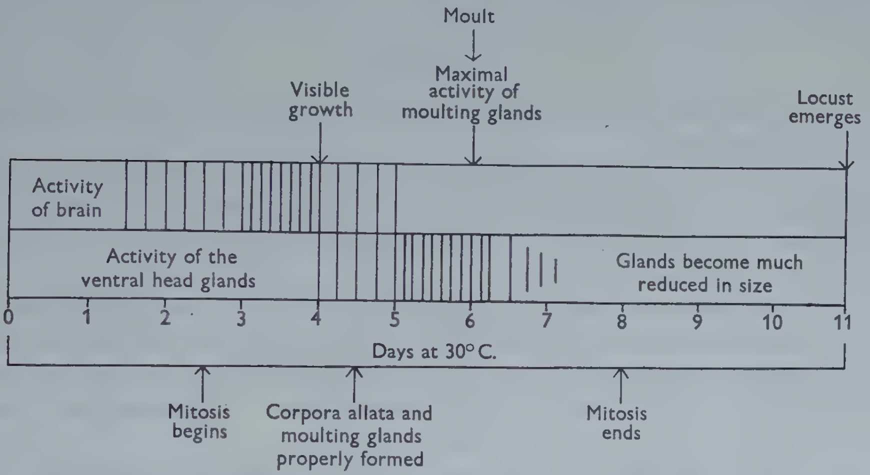
DISCUSSION

The results summarized in Text-fig. 3 show that in the embryo of *pardalina* the ventral head glands were not present as organized endocrine centres when the renewed burst of mitosis brought the quiescent phase to an end. Judging by an examination of serial sections, these glands were not formed until the 3rd day after the initiation of growth, and, according to experimental evidence in the present work, they did not reach their maximal phase of activity until the 4th day when the moult took place.

However, when mitosis began in *pardalina* large cells were present in the protocerebrum in a position corresponding to that occupied at a later stage in development by the well-defined neurosecretory cells. Yet it was inconceivable that just such cells as these in the brain were responsible for initiating growth and differentiation. Besides, Williams (1952) has demonstrated very clearly that the brain in insects is incapable of performing this function.

The evidence therefore converges convincingly in support of the conclusion, that in the embryo of *pardalina* there was no participation of organized endocrine tissue in the initiation of mitosis at the end of diapause.

Thus, the ventral head glands played no part in initiating growth at this early stage of development in *pardalina*, but on the other hand, as shown by the results of the ligaturing experiments, they were responsible for inducing the embryonic moult. An important point arising from these observations was that the epidermis was activated 3 days before the glands were formed and 4 days before it retracted from the cuticle. Just such evidence as this has formed the principal basis for the view put forward in the present paper that the precise function of the ventral head glands in the locust embryo is to induce the retraction of the epidermis from the cuticle, an event which is essentially a morphodynamic one.



Text-fig. 3. The activity of the endocrine centres in the embryo of *Locustana pardalina* in relation to each other, to growth and to the moult during post-quiescent (post-diapause) development. The closer hatching denotes a period of maximal activity.

The next question which arises is how, in the absence of organized endocrine tissues, can the initiation of post-diapause development in the egg of *ardalina* be explained?

Matthee (1951) pointed out that in *pardalina* an uptake of water by a diapausing egg only resulted in a slight enlargement of the embryo. Yet when the egg is in the quiescent phase, which follows diapause in *pardalina*, the uptake of water with consequent turgidity is an essential prerequisite not only for the initiation of mitosis, but also for further and healthy progress in development.

The inference to be drawn from these observations is that the cells at the end of diapause in the locust egg are competent to proceed with differentiation and growth, and they will do so if suitably stimulated. The effectiveness of wetting in terminating quiescence in the egg of *pardalina* may be explained in terms of a stretching of the cells, due to the uptake of water, stimulating the nervous system which in turn is responsible for inducing further progress in growth. In view of the stretching of *Rhodnius* in feeding being necessary for stimulating the afferent nerves to produce the same effect (Wigglesworth, 1934) the above explanation is a plausible one.

The causation of diapause in the egg of *pardalina* has not been investigated. However, experimental evidence resulting from work on *Bombyx* (Fukuda, 1951, 1952) has led to the view that a diapause factor originating in the sub-oesophageal ganglion of the fertile female exercises an inhibitory influence on growth in the diapause eggs produced.

If just such a diapause factor does operate in the egg of *pardalina*, the present work indicates that when this diapause factor is no longer able to exert its influence the cells once again become competent to divide. Only then can mechanical stimulation induce the young embryo, in which at this time organized endocrine tissues do not operate, to make further progress in growth and differentiation.

SUMMARY

1. A brain-ventral head gland system operates in embryos of *Locustana pardalina* and *Locusta migratoria*.
2. The initiation of growth and differentiation on the termination of diapause in the egg of *pardalina* takes place before the ventral head glands are formed.
3. Maximal activity in the ventral head glands coincides with the retraction of the epidermis from the cuticle.
4. Embryos, dissected out of the egg, were kept alive in aerated sterile Ringer's solution for up to 2 weeks during which time they progressed in their development.
5. If post-katatrepsis embryos are ligatured between the thorax and abdomen before a 'critical' period the moult is limited to the thorax. If ligatured immediately behind the head, the body fails to moult.
6. Since on the termination of diapause in the egg of *pardalina* mitosis begins before the formation of the ventral head glands, it is suggested that in locust embryos these glands are exclusively concerned with the retraction of the epidermis from the cuticle.
7. It is suggested that the uptake of water by the egg of *pardalina* in stretching the cells stimulates a growth factor which, although present throughout the diapause phase, is only capable of initiating mitosis after the diapause phase has come to an end.

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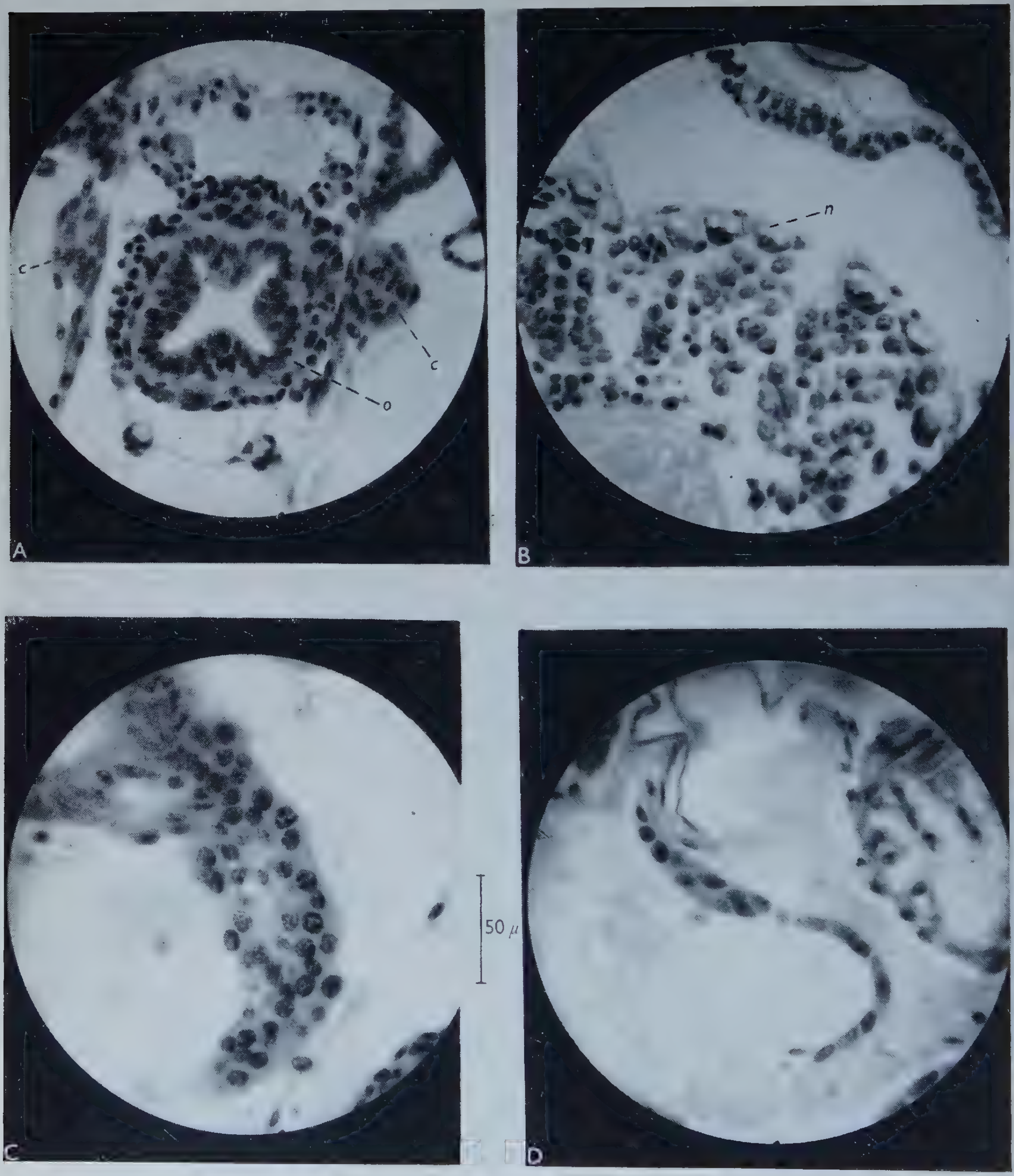

JONES—ENDOCRINE ACTIVITY DURING INSECT EMBRYOGENESIS. FUNCTION OF THE VENTRAL HEAD GLANDS IN LOCUST EMBRYOS (*LOCUSTANA PARDALINA* AND *LOCUSTA MIGRATORIA*, ORTHOPTERA)

(*Facing p.* 185)

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EXPLANATION OF PLATE

Endocrine centres in the embryonic stage of *Locustana pardalina*

A. Section of embryo on the 5th day after wetting the quiescent egg (or 3 days after the initiation of growth) showing the corpora allata on either side of the oesophagus. *c*, corpora allata; *o*, oesophagus.

B. Section of protocerebrum showing neurosecretory cells as they appeared on the 4th day after wetting the quiescent egg (or 2 days after the initiation of growth). *n*, neurosecretory cell at the periphery of the protocerebrum.

C. Section of part of the ventral head gland during its period of maximal activity on the 6th day after wetting the quiescent egg (4 days after the initiation of growth).

D. Section of part of a ventral head gland (corresponding to that shown in C) immediately before emergence.

SOME ASPECTS OF THE RESPIRATION OF SIX SPECIES OF FISH FROM UGANDA

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(*Received* 5 *September* 1955)

The present study is an attempt to correlate ecological data with certain aspects of the respiration of six species of fish typical of local waters. The species of fish examined were:

Lates albertianus Worthington	*Bagrus docmac* Forsk.
Tilapia esculenta Graham	*Clarias mossambicus* Peters
Mormyrus kannume Forsk.	*Protopterus aethiopicus* Heck.

METHODS

The fish were collected from gillnets or seine nets and usually taken alive to the biological laboratory at Makerere College. Here blood samples were taken which, if not used immediately, were stored below 0° C. In some cases the fish were bled when they were caught and the blood stored in a vacuum flask containing an ice-salt mixture. The blood was collected from an afferent vessel in the gill arch or from the caudal artery. In the case of *Clarias* and *Protopterus*, the blood was taken from the ventricle of the heart. Specimen tubes of 1 or 5 ml. capacity were cleansed and, before sterilization by dry heat, suitable quantities of Wintrobe's solution (an anticoagulant containing oxalate) were added. The blood from the fish flowed directly into these tubes and, when the required quantity had been collected, the tube was stoppered, shaken vigorously to dissolve the oxalate crystals and stored. Any tubes in which the blood had clotted were discarded. (It was noted that the blood of *Tilapia* clotted more readily than that of other fish examined. Several specimens often had to be used before a satisfactory sample was obtained.)

The blood was diluted with approximately three parts of distilled water, and the clear supernatant solution of haemoglobin used in a tonometer of the bulb-capillary type (Fox, 1945). Gas mixtures were made up in a manometer which incorporated the gas pipette from the Haldane apparatus and consisted of an inert gas, oxygen and carbon dioxide in carefully measured proportions. The gas, inert from the point of view of respiration, was either nitrogen or hydrogen. This was freed from all traces of oxygen by washing with an alkaline solution of 1-2-4 triacetoxybenzene and subsequently by passing through a copper gauze furnace. Air, from which all traces of carbon dioxide were removed by soda-lime, was used as a source of oxygen. Carbon dioxide was obtained from cylinders.

The gas mixture was flushed through the tonometer which was then rotated slowly for 20 min. to allow time for equilibration at room temperature. This varied between 23 and 24° C. The spectrum of the solution, seen through a Zeiss microspectroscope, was then compared with that of human blood at varying degrees of oxygenation (R. Hill, quoted by Fox, 1945). A 'Crista' comparator was modified so that the spectrum of the standard blood at any degree of oxygenation could be observed in the same field of view as that of the experimental solution of haemoglobin. The haemoglobin content of the fish blood was estimated in a 'Beckman' haemoglobinometer.

Data from five specimens of *Tilapia*, three of *Mormyrus* and two of *Lates* were used in the construction of dissociation curves. The other curves are from measurements using blood from single specimens.

RESULTS

Lates albertianus

Members of this species, which is endemic to Lake Albert, are amongst the largest fresh-water fish. It may be seen from the graphs in Fig. 1 that the presence of carbon dioxide considerably reduces the capacity of the haemoglobin for combining with oxygen. The haemoglobin is 50% saturated at a pressure of 17 mm. oxygen, but in the presence of 25 mm. carbon dioxide, the oxygen pressure has to be doubled in order to reach the same saturation figure. It is clear that this fish requires water with a high dissolved oxygen and low carbon dioxide content. A comparison with the dissociation curves obtained from other fish (Figs. 2–6) shows that the haemoglobin in *Lates* blood has the lowest affinity for oxygen. These data show that, in the absence of carbon dioxide, the haemoglobin cannot be fully saturated unless the lake water in contact with the gills contains at least 5 p.p.m. dissolved oxygen. Such requirements are reflected in its distribution. The waters of Lake Albert are clear, well stirred by the wind and have a very small biological oxygen demand. Dissolved oxygen is likely to be always near saturation point and the carbon dioxide content low. Similarly, other species of this genus are found in waters where the oxygen concentration is unlikely to fall or that of carbon dioxide to rise to toxic levels.

This physiological dependence on well-aerated water may offer an explanation for the occasional heavy mortalities, limited to this species, which are reported from Lake Albert (see also Worthington, 1929).

An extensive mortality occurred in November 1951, and dead *Lates* were washed ashore in large numbers. Samples were carefully examined, and the available evidence indicated that the most likely cause of death was asphyxiation. The hydrological data below provides a possible explanation.

The main inflow to Lake Albert is the Semliki river which comes from Lake Edward but also has many tributaries from the Ruwenzori and Eturi Forest regions. The examination of water samples, collected at frequent intervals during 1953–4, shows that these tributaries carry water which is relatively cool and rich in reducing substances. Data collected by the Hydrological Survey Department (1951) show that, during August 1951, the tributaries supplied an exceptionally high proportion of the total discharge of the Semliki River into the lake. For this short period therefore, the inflowing water, being cooler than the lake water, would tend to flow along the floor of the lake and remain a disparate layer until a sudden storm caused mixing. Gill-breathing fish in these areas would then be adversely affected by the rapid, though probably short-lived, reduction in dissolved oxygen and increase in carbon dioxide.

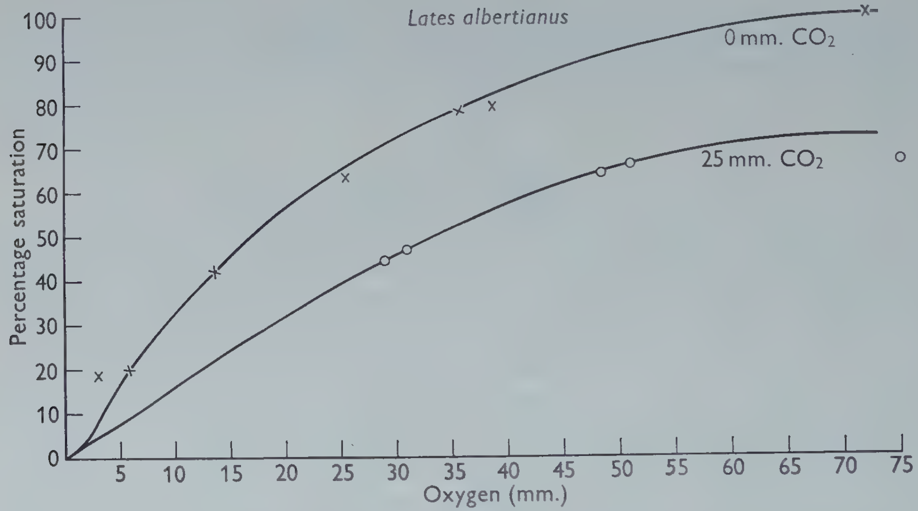

Fig. 1. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Lates albertianus*. The average haemoglobin content of the blood used in these estimations was 9·1 g./100 ml.

It was remarkable that over 70% of the dead fish were over 50 lb. in weight, many over 200 lb. and only about 1% were less than 10 lb. in weight. It seems likely that the larger fish were more closely dependent on optimum conditions of aeration than the smaller specimens.

L. albertianus has been found in the Victoria Nile as far upstream as Fajao and in the Albert Nile as far downstream as Pakwach (Worthington, 1929). The spread of this species upstream is clearly limited by the Murchison Falls at Fajao. It seems likely that its spread downstream may be limited by unfavourable conditions of aeration of the water, for recently collected data have shown that, as the Nile flows through the Sudd (a papyrus swamp region extending for about 300 miles), the carbon dioxide concentration in the water rises considerably. The dissolved oxygen content of the water was also found to drop, on occasions, to very low values (Hydrobiological Research Unit, 1954; J. F. Talling, personal communication).

Tilapia esculenta

From a commercial point of view, this is the most important fish in Uganda and large numbers are caught annually in Lake Victoria. *Tilapia esculenta* is found in shallow waters which support a good growth of plankton. Dissolved oxygen concentrations are usually high, but may drop to about 50% saturation. These coastal waters are frequently bounded by swamps and have a soft muddy bottom. Carbon dioxide in solution is often present, especially after heavy rain when swamp water may be flushed out by inflows into the lake water. The dissociation curve in the absence of carbon dioxide (Fig. 2) has a steeper slope than that of *Lates*. The haemoglobin is fully oxygenated at tensions which would barely oxygenate that of *Lates* to a value of 30%. *Tilapia* is unlikely, therefore, to suffer from anoxia under conditions which would cause *Lates* severe respiratory distress.

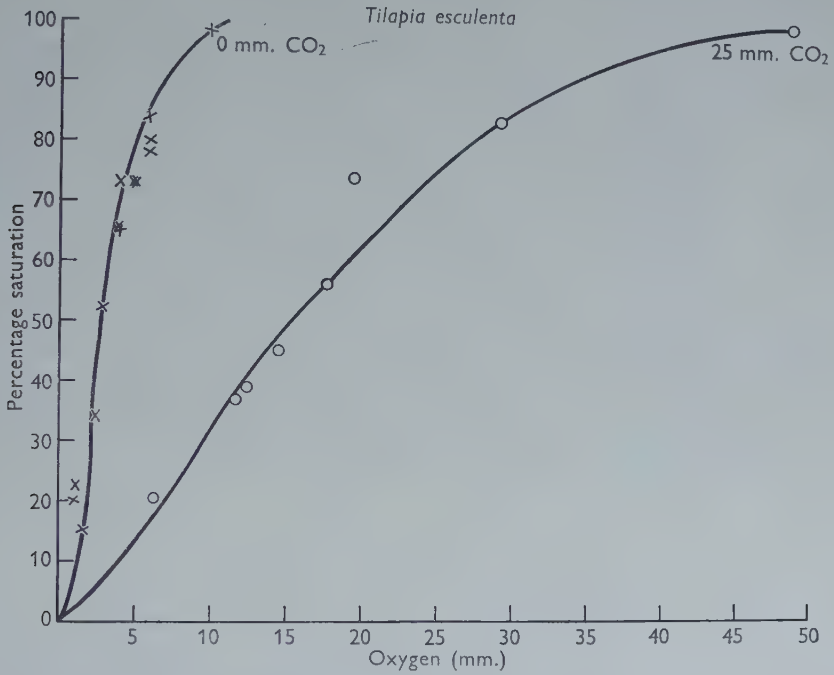

Fig. 2. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Tilapia esculenta*. The average haemoglobin content of the blood used in these estimations was 7·5 g./100 ml.

Occasionally, large mortalities of fish have been reported from Lake Victoria, but details are rarely available (Graham, 1929). A local mortality of fish, however, was reported from Entebbe on 31 July 1953. The fish affected consisted almost entirely of *Tilapia* and *Haplochromis* species. Examination produced no evidence of disease, and local fishermen collected large numbers of dead and dying *Tilapia* for subsequent sale. A day or so prior to the first reports of this mortality there was an exceptionally strong offshore wind at Entebbe. Such a wind would cause a movement of surface water away from the shore and there would be a corresponding movement inshore of water from the deeper layers in the lake. Temperature records for July show that the density differences between surface and bottom waters in the lake a few miles offshore were small and so relatively little energy would be required to cause a movement of large amplitude of the water strata within the lake. The available data indicate that the cause of this mortality was asphyxiation due to a limited upwelling of poorly aerated water. The difference in slope of the dissociation curves shows that *Mormyrus* and *Bagrus* are not as sensitive to poor aeration as *Tilapia*. This may explain why no members of these genera were found amongst those affected by this mortality.

Samples of the haemoglobin of *Tilapia esculenta* and of *Mormyrus kannume* were exposed experimentally to tensions of 50 and 250 mm. Hg of carbon dioxide. No oxyhaemoglobin could be detected in such solutions at oxygen tensions of up to 30 mm. Hg. It may be significant that the haemoglobin of *Tilapia* and *Mormyrus*, after exposure to these tensions of carbon dioxide, behaved abnormally when tested subsequently in oxygen mixtures containing no carbon dioxide. The carbon dioxide gas mixture was flushed out of the tonometer containing the haemoglobin solution by a stream of air, but subsequent observations showed that oxyhaemoglobin, if formed at all, was rapidly broken down again. Powers *et al.* (1932) found a similar inactivation effect when working on the blood of *Cycleptus elongatus* (Le Sueur) and of *Stizostedion vitreum* (Mitchell). This phenomenon may provide an explanation for the observation that, during a mortality, the affected fish are dying even when they have drifted into well-aerated water where others of the same species are suffering no ill-effects.

Mormyrus kannume

This species occurs throughout the Nile system of lakes and rivers. Its food often consists largely of lake-fly larvae (e.g. Chironomidae) from the bottom deposits. Hydrological records, collected over a number of years, have shown that Lake Victoria is thermally stratified for long periods annually. At these times, dissolved oxygen concentrations in the bottom waters fall to very low values (Fish, unpublished). *Mormyrus* is therefore exposed to low oxygen tensions whilst foraging very near or in the mud surface. The slope of the dissociation curve (Fig. 3) shows that it is well adapted to withstand such conditions. The haemoglobin concentration in the blood is high, and it is probable that, from the red colour of the muscle tissues, there may also be a high content of myoglobin. It is possible that the relatively high content of haemoglobin and myoglobin in this fish may have an oxygen storage function and serve to buffer the effect of very adverse conditions of aeration that may be encountered when feeding close to the mud surface.

Bagrus docmac

This fish is found throughout the Nile system. Graham (1929) found that, in Lake Victoria, it attained its largest size in the deeper waters but was by no means limited to any particular depth, being caught from 14 to 130 ft. The slope of the dissociation curve (Fig. 4) shows that *Bagrus* can successfully withstand conditions of low oxygen tension. It is of interest that Graham (1929), recording the stomach contents of 170 specimens which contained food, showed that 17% of them contained nothing but prawns (*Caridina* species). A few personal observations have indicated that these may be the only animals within the food range of *Bagrus* to be found in the deeper waters of Lake Victoria, which are poorly aerated at certain times (see above).

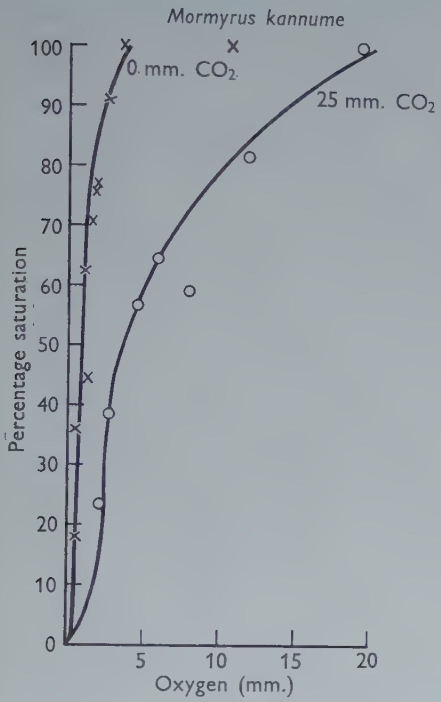

Fig. 3

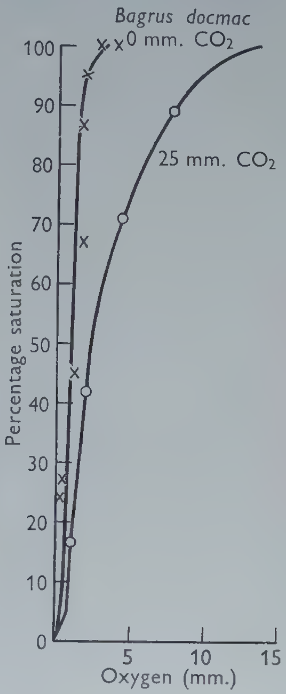

Fig. 4

Fig. 3. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Mormyrus kannume*. The average haemoglobin content of the blood used in these estimations was 11·7 g./100 ml.

Fig. 4. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Bagrus docmac*. The average haemoglobin content of the blood used in these estimations was 6·9 g./100 ml.

Clarias mossambicus

This fish is found in many of the African lakes and swamps and is common in Lake Victoria. It is found at all depths from swamps to over 200 ft. of water, and has been seen moving through damp grassland, although it is not possible to say whether these terrestrial excursions are primarily for food or migrations from one locality to another (Welman, 1948). This wide range of habitat is only possible to the fish by virtue of its unusual respiratory mechanism. It depends largely on the oxygen in the air for its respiration and can be asphyxiated if access to air is prevented. The dissociation curve (Fig. 5) in the absence of carbon dioxide is much steeper than that for a fish limited to the open water, such as *Lates*. Such a curve is advantageous, for *Clarias*, when leading a purely aquatic life, can only take in a limited volume of air at relatively infrequent intervals.

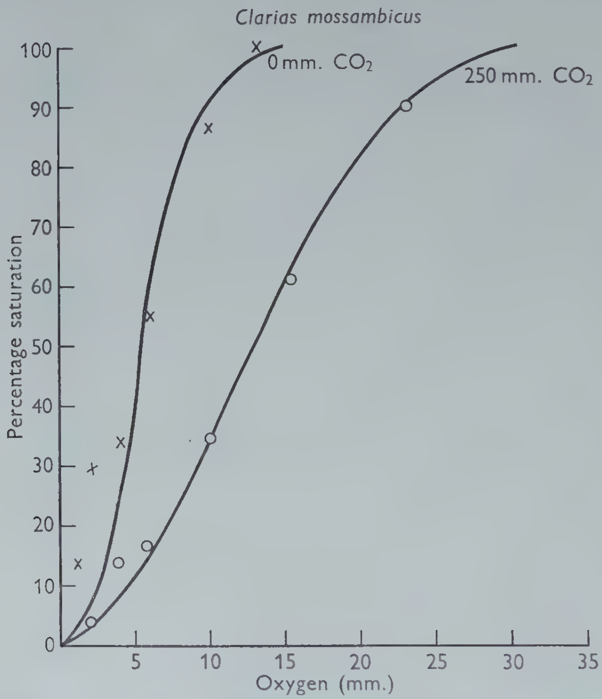

Fig. 5. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Clarias mossambicus*. The average haemoglobin content of the blood used in these estimations was 13·3 g./100 ml.

In comparison with the other fish studied, its blood pigments were found to be remarkably insensitive to carbon dioxide. This property of the haemoglobin is very important in view of the accumulation of carbon dioxide in the air surrounding the supra-branchial organ during the intervals between 'breathing'. This fish is also exposed to high concentrations of carbon dioxide in its swamp habitat.

Protopterus aethiopicus

This lungfish is common in the Upper Nile Region and Lake Victoria. It is found in shallow water and swamps and breathing is effected by means of lungs. The dissociation curve (Fig. 6) is similar to that obtained for *Clarias* in having the same tolerance to very high carbon dioxide tensions. The main difference between the two curves, according to the present data, is that a steeper slope is found in the case of *Clarias*.

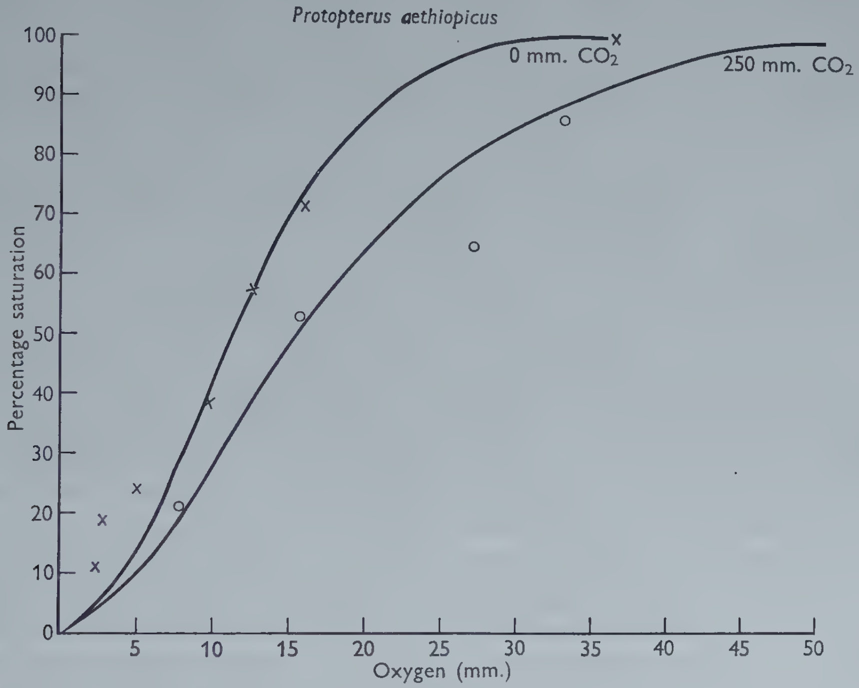

Fig. 6. Oxygen dissociation curves for haemoglobin solutions prepared from the blood of *Protopterus aethiopicus*. The average haemoglobin content of the blood used in these estimations was not determined.

DISCUSSION

It is not unexpected, in view of the variability of aquatic habitats in Uganda, to find large differences in the shape and slope of the dissociation curves for these species of fish. However, Willmer (1934), working with the whole blood of fish in British Guiana, found that the curves, in the absence of carbon dioxide, were similar in all the fish he examined although they came from habitats differing widely in conditions of aeration. The blood from these fish differed widely in response to carbon dioxide, and this response was correlated with habitat. The largest Bohr effect was found in the open-water type of fish, e.g. the paku (*Myleus setiger*, Mull & Troschel) and the smallest in the air-breathing swamp fish, e.g. the electric eel (*Electrophorus electricus*) and the hassa (*Hoplosternum littorale* Hancock). He found no evidence to suggest that the blood of those fish living in swamps was adapted for complete saturation at low oxygen tensions, although such adaptation had been found in carp, pike and eel (Krogh & Leitch, 1919).

It must be remembered, however, that these air-breathing fish live in shallow water and are therefore unlikely to be exposed to low oxygen tensions. The fish investigated in this paper are found frequently in deep water and are all lake fish or potentially so. Easy access to the air may not, therefore, always be possible, and their blood may often be exposed to low oxygen tensions. Willmer also noted that fish blood in British Guiana is subjected to tropical temperature (28° C.) and suggested that, under similar conditions, such blood might easily behave like carp blood if subjected to the lower temperature (15° C.). The difference between whole blood and haemoglobin solutions was investigated by Root, Irving & Black (1939), who found that the response to carbon dioxide of whole and haemolysed blood from the same individual fish could be quite different, although the degree of difference varied between species. Bearing these limitations in mind, certain broad conclusions may be drawn relating the dissociation curves for the fish selected here to their ecology.

The haemoglobin from the blood of *Lates* has a slight affinity for oxygen and shows a considerable Bohr effect in contrast to that of the other species examined. The competence of the haemoglobin as an oxygen collector is low, but as a result, the gas is more readily released in the body. This makes for the efficient cellular metabolism essential for such a fast and powerful predator, but the respiratory surfaces must be bathed in water containing high tensions of dissolved oxygen and negligible carbon dioxide for the haemoglobin to be fully saturated. The haemoglobin of *Mormyrus* and *Bagrus* is saturated at low oxygen tensions and therefore takes up oxygen as efficiently in poorly as in well-aerated water. The range of such fish is greatly extended as a result. A high sustained level of activity may not, however, be possible, because the tension of oxygen within the tissues must be comparatively low in order to affect transfer from the haemoglobin. The adverse effect of this oxygen tension in the tissues is often modified in various ways. It is of interest in this connexion to note that, of the fish examined here, those with the steeper curves have the higher haemoglobin contents in the blood.

Carter (1955) has shown that there are large bodies of water in Uganda which are impossible habitats for gill-breathing fish owing to the low dissolved oxygen and the high carbon dioxide concentration present. Certain species of fish are known to survive under such conditions by utilizing the oxygen dissolved in the surface layers of water (G. E. Hutchinson, unpublished material quoted by Allee, Emerson, Park, Park & Schmidt, 1950; Carter & Beadle, 1931). Apart from such fish, only those able to utilize the oxygen in the air are found in these poorly aerated waters, namely, *Clarias* and *Protopterus*. The very small Bohr effect shown in the dissociation curves of both these fish is necessary for respiration in air-breathing organs which always contain carbon dioxide (Carter, 1931).

Data, collected by American workers on species of Siluroidea, are of interest in this connexion. Prosser, Bishop, Brown, John & Wulff (1952) note that the

American catfish has been found to be able to endure tensions of carbon dioxide of the order of 400–500 mm. Hg for long periods. Black (1940) has also found that the blood of *Ameiurus nebulosus* (Le Sultar) combines a high affinity for oxygen with a low sensitivity to the presence of carbon dioxide.

SUMMARY

The oxygen dissociation curves of the haemoglobin of six species of fish from Uganda, and the effect of 25 mm. Hg of carbon dioxide on them are presented graphically. The fish were selected from habitats varying widely in contents of dissolved oxygen and carbon dioxide. The ecology of these species is correlated with differences in the shape and slope of the curve. Records of fish mortalities in Lakes Albert and Victoria are examined in the light of the new data available and suggestions as to the causes are put forward.

This work would not have been possible without the assistance of Prof. L. C. Beadle, Makerere College, Kampala, Uganda. He constructed the apparatus used, made all the estimations of haemoglobin of fish blood, and freely placed his wide knowledge and experience at my disposal. I am grateful, also, to the staff of the Fisheries section of the Game and Fisheries Department of Uganda and that of the East African Research Organization, in particular Mr P. H. Greenwood, for discussion and help in collecting and identifying fish samples. Water samples from the Semliki River were collected by the Department of Hydrological Survey, Uganda.

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GLYCOGENOLYSIS IN THE LIVER OF THE COMMON FROG, *RANA TEMPORARIA*

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(*Received 22 July* 1955)

INTRODUCTION

It has been known for many years that the metabolism of the frog is subject to seasonal variations. Liver glycogen and total body glycogen attain a maximum value in autumn, and a minimum in spring (Athanasiu, 1899; Pfluger, 1907; Kato, 1910; Bleibtreu, 1911; Goldfederowa, 1926). A few seasonal estimations of blood sugar were reported (Lesser, 1913; Slome, 1936), but the first comprehensive study of the problem was conducted by Smith (1950), who investigated the seasonal changes in blood-sugar level, fat-body development, liver-glycogen content and gonad condition in *Rana temporaria*.

In view of these seasonal changes, it was felt that temperature might be an important factor in the regulation of amphibian carbohydrate metabolism. It was therefore decided to investigate the fundamental relationship between temperature and glycogenolysis in the frog's liver, with a view to establishing the role of changing environmental temperature in the observed seasonal changes in carbohydrate metabolism. For this purpose a tissue-slice technique had many advantages, as the number of variables can be greatly reduced. For example, by incubating slices from the same liver at different temperatures, not only can individual variation be controlled, but simultaneous readings over a wide temperature-range are obtainable. Such a technique also lends itself to the *in vitro* study of the action of drugs and hormones on glucose production.

MATERIALS AND METHODS

Materials

Frogs were either collected personally in the Wirral Peninsula, or sent in a fresh condition from collectors in Staffordshire and the Norwich area. All frogs were sexually mature and were kept in a tank-room, which was well ventilated and at approximately atmospheric temperature. The daily maximum, minimum and actual temperatures of the tank-room were noted each time a frog was killed. Newly arrived frogs were allowed to settle down in the tank-room for at least a day before being used.

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Apparatus

As it was necessary to work below room temperature, an incubation apparatus was constructed in a cold-room maintained at a constant temperature of 3° C. The apparatus consisted of six constant-temperature baths thermostatically controlled at approximately 7, 10, 12·5, 15, 18 and 21° C., respectively, and the temperature of each bath was constant to within ± 0·1° C. during each experiment. Each water-bath was mechanically stirred, and a flask-carrier (with provision for two flasks per bath), oscillated above the baths.

Methods

The heaters and stirrers of the apparatus were switched on 2 hr. before an experiment was due to begin. A frog was pithed in the tank-room, with minimal struggling by the animal, immediately removed to the cold-room where the liver was excised and dropped into chilled Ringer (pH 7·6). Each lobe of the liver was blotted dry, sliced between ground-glass plates, and the slices (approximately 0·2 mm. thick) dropped into chilled Ringer. A 25 ml. flask containing exactly 5 ml. of Ringer was placed in the appropriate bath 15–30 min. before starting an experiment, and gassed with oxygen for 2 min. immediately prior to the addition of liver. Accurately weighed liver slices (30–50 mg.) were introduced into each flask in turn, and the flasks stoppered. In the case of controlled experiments the same procedure was carried out for a second set of flasks, each containing 5 ml. of the experimental medium. The time of addition of liver to each flask was noted to within 30 sec. In all experiments a liver slice was weighed and analysed for glycogen. The temperatures of the baths were checked at the beginning and end of each experiment. Exactly 2 hr. after the addition of liver slices, each flask was removed in turn from the apparatus, and a 2 ml. aliquot withdrawn into a graduated centrifuge tube.

Estimation of glucose

The 2 ml. aliquots from the incubation flasks were deproteinized by the method of Levvy (1946). After centrifugation 5 ml. of the supernatant fluid were withdrawn from each tube for glucose estimation (Hagedorn & Jensen, 1923). Glucose production was calculated as a percentage of the wet weight of liver used in each flask. A blank, in which 2 ml. Ringer was substituted for the 2 ml. of incubation medium, was treated in the same way, and when drugs or other agents were employed in the experimental Ringer, a sample of this medium was also carried through as the experimental blank.

Estimation of glycogen

A weighed liver slice was dropped into approximately 1 ml. of 30% potassium hydroxide solution in a graduated centrifuge tube, and analysed for glycogen by the method of Good, Kramer & Somogyi (1933). The results were expressed as a percentage of the wet weight of tissue.

Incubation medium

The Ringer's solution used in this work was of the following composition: 0·65% NaCl, 0·014% KCl, 0·012% $CaCl_2$, 0·001% NaH_2PO_4. Immediately before use the pH was adjusted to 7·6 by the addition of the necessary amount of a strong solution of Na_2HPO_4. Glass-distilled water was used throughout.

Drugs used. Adrenaline: adrenaline B.P.; *noradrenaline*: Bayer 'Levophed'; *thyroxine*: sodium thyroxine (B.D.H.); *insulin*: insulin (B.D.H. 40); *ergotamine*: Sandoz 'Femergin'; *dibenzyline*: dibenzyline (Smith, Kline and French) (made up as follows: dibenzyline, 5·0 g.; absolute alcohol, 48·5 ml.; propylene glycol, 48·4 ml.; hydrochloric acid, 0·2%).

RESULTS

Experiments with unmodified Ringer

The glucose produced by 100 mg. of liver in 2 hr. at each of the six temperatures was calculated. These six glucose values were plotted against the incubation temperatures to give a temperature-glycogenolysis curve, and it was found that the curves could be divided into two main groups:

(1) Those curves in which the increase in glycogenolysis with rise in temperature was linear over the range 7–21° C.

(2) Those curves where the rates of glycogenolysis lay on two straight lines intersecting at any point between 10 and 18° C., but most frequently between 12 and 14° C. The rate of glycogenolysis increased progressively from 7° C. to about 13° C., but further rise in temperature caused a disproportionate increase in glucose production. Smith (1951, 1952*a*) obtained a similar type of curve for the rate of beat of the isolated perfused frog's heart, and the temperature-glycogenolysis curves showing this sharp increase in rate above a critical temperature have been termed type *B* to conform with Smith's terminology.

The temperature coefficient (Q_{10}) for the overall glucose production from 7 to 21° C. was calculated for all the temperature-glycogenolysis curves. In addition, the Q_{10} of the line joining the 7 and 10° C. values of the type *B* curves, extrapolated to 21° C., was calculated. A frequency histogram was constructed from the Q_{10} values of all linear curves. This showed two peaks at 1·65 and 1·95, with a minimum at 1·75, and the linear curves were divided into two groups on this basis. Those with a Q_{10} greater than 1·75 were termed high linear, and those with a Q_{10} less than 1·75 were termed low linear.

The average high linear, low linear and type *B* curves were obtained by reading off the glucose production at 7, 10, 12·5, 15, 18 and 21° C. from each particular curve in that group, and calculating the mean. The data for each group, together with the number of livers, and the temperature coefficients, are given in Table 1, on which Fig. 1 is based. A few curves were obtained which could not be assigned to any of the three types described above. Some showed a sudden small rise in glucose production at a particular temperature, in an otherwise linear or type *B* curve, and a few curves appeared which had an extremely low glucose production at all temperatures.

Table 1. *Mean rates of glucose production* (± s.e.) *at different temperatures, and temperature coefficients* (Q_{10}) *for the three types of temperature-glycogenolysis curve*

Type of curve	No. of livers	Mean rate of glucose production expressed as mg. glucose/100 mg. liver/2 hr. ± s.e. at						Q_{10} (7–21° C.) ± s.e.
		7° C.	10° C.	12·5° C.	15° C.	18° C.	21° C.	
Low linear	13	0·51 ± 0·040	0·59 ± 0·051	0·65 ± 0·060	0·73 ± 0·070	0·81 ± 0·082	0·89 ± 0·096	1·48 ± 0·06
High linear	8	0·45 ± 0·041	0·60 ± 0·054	0·72 ± 0·057	0·85 ± 0·074	1·02 ± 0·083	1·16 ± 0·087	1·99 ± 0·05
Type *B*	68	0·48 ± 0·014	0·56 ± 0·017	0·63 ± 0·017	0·76 ± 0·023	0·94 ± 0·031	1·18 ± 0·033	1·94 ± 0·04 *1·45 ± 0·04

* Value calculated from the line obtained by extrapolation of the 7 and 10° C. rates to 21° C. (see text).

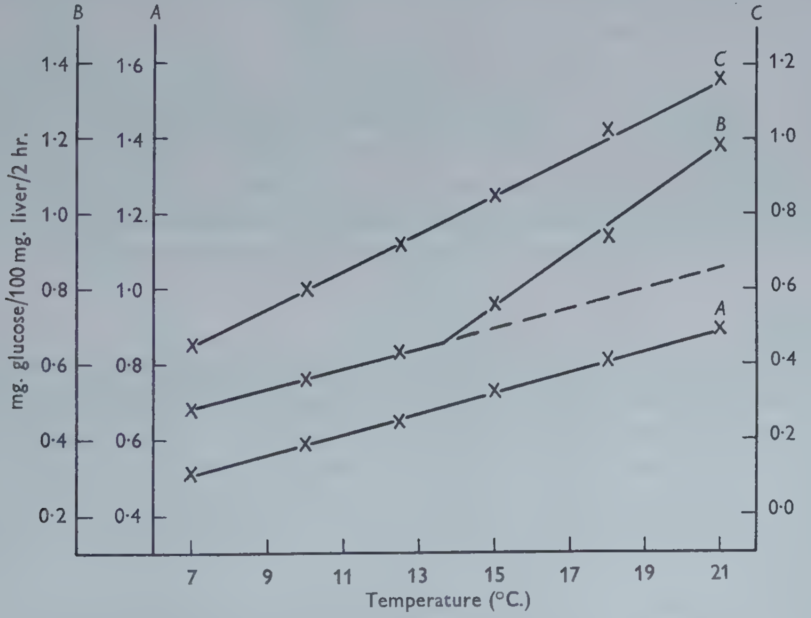

Fig. 1. Various forms of the temperature-glycogenolysis curve observed during 1952–3. ×——×, mean values at the stated temperature-intervals (from Table 1); – – – –, projection of the line joining the 7, 10 and 12·5° C. values to 21° C. *A*, mean low linear curve; *B*, mean type *B* curve; *C*, mean high linear curve.

From Table 1 it will be seen that there is no significant difference in the average glucose production at 7° C. for all three types of curve, and that the 21° C. glucose values of the type *B* and high linear curves are very similar. The mean high linear, low linear and type *B* curves had Q_{10} values of 1·99, 1·48 and 1·94 respectively, and the Q_{10} of the lower portion of the type *B* curve extrapolated to 21° C. (1·45) was almost identical with that for the mean low linear curve (1·48), so that when these two latter lines are drawn with the same co-ordinates, they are almost coincident. There is no significant difference between the Q_{10} values of these two lines, or between the Q_{10} values of the high linear and type *B* curves. However, the difference between the Q_{10} values of the low linear and high linear, and the low linear and type *B* curves is highly significant ($P < 0{\cdot}001$ in each case).

During 18 months of experimental work, there were 68 type *B*, 13 low linear and 8 high linear curves. Type *B* curves were obtained in every month of the year, the low linears appeared mainly during the period February–April, and sporadically at other times, while the high linears occurred irregularly throughout the year. The few curves which showed a very low glucose production at all temperatures occurred, with the exception of two cases, in March and April, during the spawning and post-spawning period. Liver-glycogen content was low at these times (1–3 %), and it was observed that when the liver-glycogen content was less than 1 %, glucose production was reduced at all temperatures. With higher liver-glycogen values of 2 % upwards, glucose production was not restricted.

Experimental results

The pattern of the type *B* curve, which indicated a disproportionate increase in glucose production above a critical temperature, suggested a potentiation of some glycogenolytic agent. In view of this, it was decided to investigate the action of various drugs and endocrine preparations on the liver, both *in vivo* and *in vitro*. Except where otherwise stated, the control and experimental slices were obtained from the same liver, to eliminate individual variation between animals.

Adrenaline

One set of liver slices was incubated for 2 hr. with freshly prepared adrenaline-Ringer (1 in 10^7), and the control slices with unmodified Ringer. A total of six experiments were carried out during January and June. The glucose values at 7, 10, 12·5, 15, 18 and 21° C. were read off from each curve and the mean curve calculated. These data, together with Q_{10} values and standard errors, are given in Fig. 2. The 7° C. value of the control curve in Fig. 2 is considerably higher than that for the mean curves shown in Fig. 1, and is very close to the value at 7° C. obtained after adrenaline. It was thought that the relatively high level of glucose production shown by the control slices at this temperature might possibly be due to the presence of circulating adrenergic material in the animal at the time of sacrifice. If any such active material was carried over in the liver slices, then perhaps its destruction would be so retarded at the lowest temperatures that it would continue to stimulate glycogenolysis and lead to an enhanced glucose value at this end of the temperature-range.

If the assumed 7° C. control value is used (shown by dotted line in Fig. 2), then the Q_{10} values of the curves with and without adrenaline are almost identical, as adrenaline increased the rate of glucose production of liver slices at all temperatures. Fig. 2 also shows that the critical temperature at which rapid increase of glycogenolysis occurs is lower in the presence of adrenaline (12·5° C. compared with 15° C.). Other experiments were carried out with adrenaline in January, February, July and October in connexion with other work, without corresponding unmodified Ringer controls. As a matter of interest, the mean curve was calculated for a total of fifteen experiments made in the presence of adrenaline (including those described above), and the curve thus obtained is shown in Fig. 2. This latter curve was nearer the high linear form than that based on data from only six experiments, and again, this might be regarded as evidence that the extra glucose production commenced at a lower temperature (in this case in the neighbourhood of 7° C.) in the presence of adrenaline. This mean curve had a Q_{10} value of 2·08, which is in good agreement with the value of the mean high linear curve in Fig. 1 (1·99).

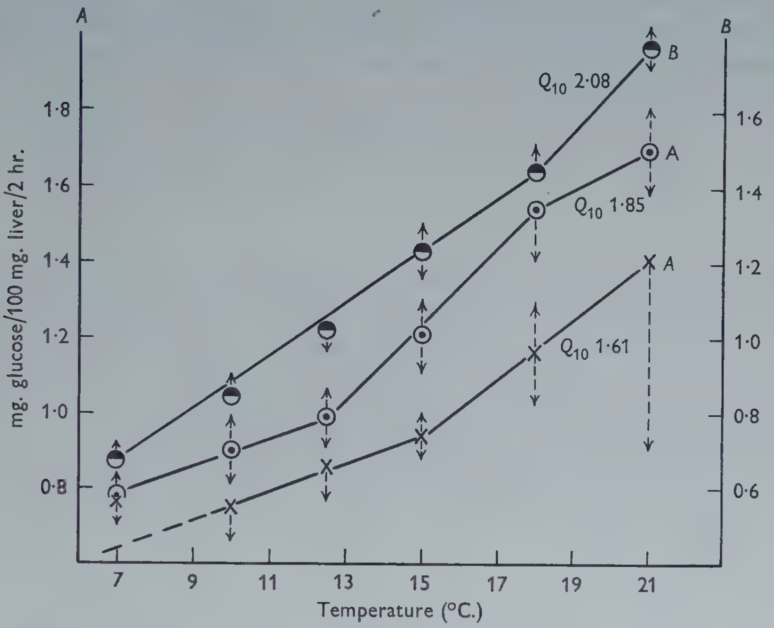

Fig. 2. The effect of adrenaline on the temperature-glycogenolysis curve. ×——×, mean glucose production of liver slices incubated in unmodified Ringer; ⊙——⊙, mean glucose production in Ringer containing adrenaline (1 in 10^7); ◓——◓, mean glucose production of all experiments using adrenaline (1 in 10^7), some without corresponding unmodified Ringer controls (see text). The vertical broken lines represent the standard errors of the mean. ----, projection of the line joining the values at 10 and 12·5° C. to 7° C. (see text).

Noradrenaline

Eight experiments were carried out during February, March and September. Noradrenaline bitartrate (1 in 10^6) raised the glucose production of the liver slices in all except one case. The results obtained were averaged, and the data, shown graphically in Fig. 3, show that noradrenaline increased the glucose production at all temperatures from 7 to 21° C. The 12·5, 18 and 21° C. values of the curve obtained in the presence of noradrenaline are joined directly, because the higher mean value at 15° C. was due to a single experiment (such irregular curves have been mentioned in the previous section). The Q_{10} of the experimental curve (1·89) is fairly close to that of the control (1·96), indicating that noradrenaline had proportionally the same effect at all temperatures. In Fig. 3, the break in the mean control curve occurs at 16·5° C., while the break in the mean curve obtained in the presence of noradrenaline occurs at 10° C. This indicates that, as in the case of adrenaline, noradrenaline leads to the earlier appearance of the extra glucose production associated with a type *B* curve.

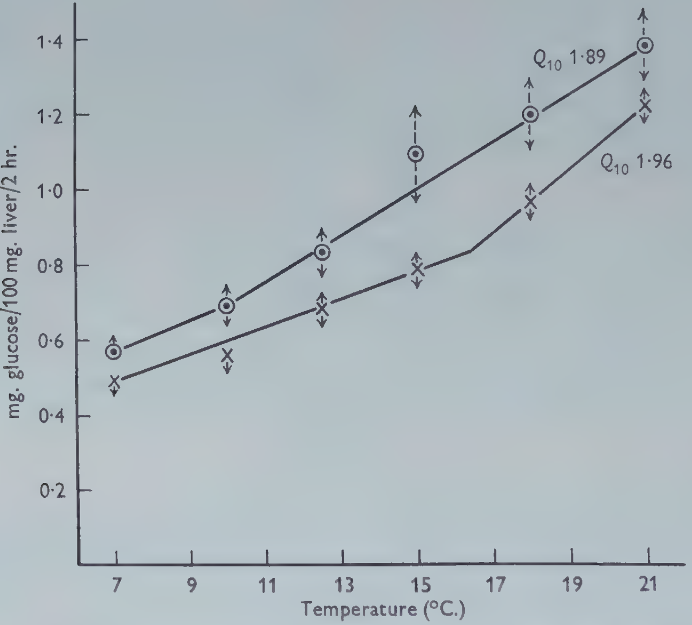

Fig. 3. Effect of noradrenaline bitartrate (1 in 10^6) on glucose production of frog's liver slices. ⊙——⊙, glucose production of slices incubated with noradrenaline bitartrate (1 in 10^6); ×——×, glucose production of slices incubated with unmodified Ringer. Mean of eight experiments. Vertical broken lines represent the standard errors of the mean.

Thyroxine

The work of Smith (1950, 1954) suggested that thyroxine might be an important factor in the carbohydrate metabolism of the frog. In the present work, liver slices were incubated with Ringer plus thyroxine (1 in 10^6) and unmodified Ringer. Thyroxine only affected glucose production in one experiment, where it converted a type *B* curve to the high linear form. This result could not be repeated, and it was concluded that thyroxine had no action on the glucose production of the frog's liver *in vitro*.

The effect of thyroxine *in vivo* was then investigated. Injections were made into the dorsal lymph sac of 1 ml. of Ringer plus thyroxine (1 in 10^6), 48 and 24 hr. before sacrifice, and control animals were similarly injected with unmodified Ringer. It was found that thyroxine administered *in vivo* had no effect on glucose production over the temperature range 7–21° C.

The thyroids of all animals used in this work were examined histologically. The state of activity of the thyroid in individual frogs was not correlated either with the type of temperature-glycogenolysis curve obtained, or with the rate of glucose production. It was concluded that thyroxine does not directly influence the glucose production of frog-liver slices. This fact, however, does not mean that thyroxine may not indirectly influence glycogenolysis in the living animal, and in fact Smith (1954) has shown that thyroxine may play a significant role in governing the blood-sugar level of frogs subjected to excitement.

Insulin

Frogs were injected with four units of insulin in the dorsal lymph sac 19 hr. prior to an experiment, and control frogs were injected with the same volume of Ringer. Liver slices were taken from one animal in each group, and incubated for 2 hr. in unmodified Ringer. Three experiments were carried out, and a typical example is given in Fig. 4. Insulin administered *in vivo* lowered the glucose production of liver slices at all temperatures to well below the range of the seasonal curves in Fig. 1. An experiment carried out 2 days after the injection of four units of insulin showed that glucose production was still very low.

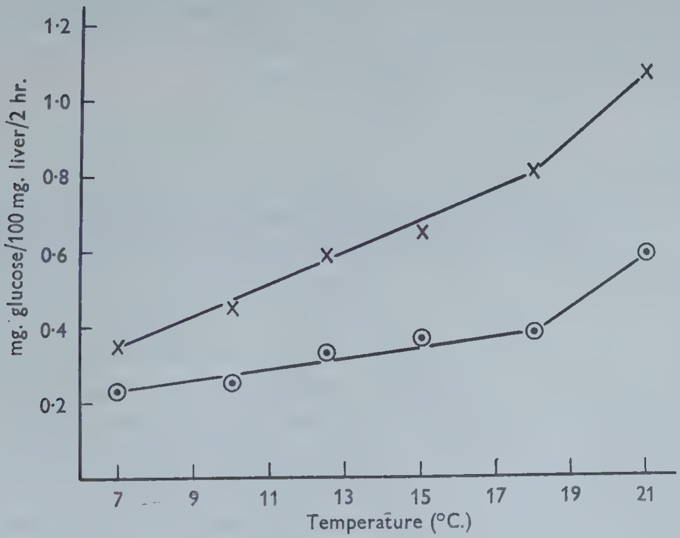

Fig. 4. Effect of *in vivo* injection of insulin on the glucose production of frog's liver slices incubated in unmodified Ringer. Female frogs, 25 March 1953. ⊙——⊙, glucose production of liver slices from frog injected with 4 units insulin 19 hr. previously; ×——×, glucose production of liver slices from control frog injected with same volume of Ringer 19 hr. previously.

The effect of insulin *in vitro* was also investigated. Liver slices obtained from a normal animal were incubated with Ringer plus insulin (10 units/litre), and with unmodified Ringer. As no decrease in glucose production was obtained, the concentration was increased to 10 units/100 ml. in subsequent experiments, but the insulin was still without effect on glucose production.

After a frog is injected with insulin, there is an interval of 4–5 hr. between time of injection and fall in blood-sugar level (Smith, 1953). In view of this, liver slices were left in contact with Ringer plus insulin (10 units/100 ml.) for 2½ hr. at 3° C. immediately prior to incubation, and the control slices were treated in the same way with unmodified Ringer. Even after this preliminary treatment, the liver slices still failed to respond to insulin *in vitro*. The slices were left in contact with their respective media at a low temperature, because it was felt that at a higher temperature they might lose a considerable proportion of their glycogen content before incubation commenced. For the same reason, this preliminary period was not extended beyond 2½ hr.

Adrenergic blockade

The present work suggested the presence of a sympathomimetic agent in the liver of the frog. In view of this, the effect of adrenergic blocking agents was investigated on liver slices incubated with otherwise unmodified Ringer. In each experiment, the liver slices were obtained from the same animal.

(1) *Ergotamine*

Slices were incubated with Ringer plus ergotamine tartrate (1 in 5×10^4) and unmodified Ringer. No decrease in glucose production was observed, and in most cases ergotamine slightly raised the glucose production at all temperatures. These experiments were repeated with added adrenaline (1 in 10^7) in both control and experimental Ringer, but no blockade was observed.

(2) *Dibenzyline*

Concentrations of either 50 or 100 mg. dibenzyline/litre were used, and the liver slices were in contact with either the dibenzyline Ringer or the control Ringer for 30 min. at 3° C. immediately prior to incubation (Harvey, Wang & Nickerson, 1952). At the end of the 30 min. period, the slices were washed in a small amount of their respective media. The experimental slices were then incubated with freshly prepared dibenzyline Ringer, and the control slices with control Ringer, *for 1 hr. only*, on account of the instability of dibenzyline in aqueous solution. In each experiment, the control and experimental slices were taken from the same liver.

Eleven experiments were carried out, seven with 50 mg. dibenzyline/litre, and four with 100 mg. dibenzyline/litre. In two cases there was no suppression of glycogenolysis, but in the remaining nine experiments glucose production was significantly lowered in the presence of dibenzyline. An experiment showing successful blockade is given in Fig. 5. The control curve was a type *B*, with a marked increase in glucose production about 12·5° C. The slices in the experimental medium produced less glucose at all temperatures compared with the controls. There is a good deal of scatter among the glucose values for the dibenzyline-Ringer, but they do approximate to the low linear type of curve, as can be seen from Fig. 5 where a straight line (Q_{10} 1·51) has been drawn between the points.

The mean curve for all eleven experiments was obtained by reading off the glucose values from each particular curve at 7, 10, 12·5, 15, 18 and 21° C. These data, together with Q_{10} values and standard errors, are given graphically in Fig. 6. The effect of temperature on the rate of glucose production was decreased in the presence of dibenzyline, and at 21° C. the experimental and control glucose values are significantly different (P less than 0·01).

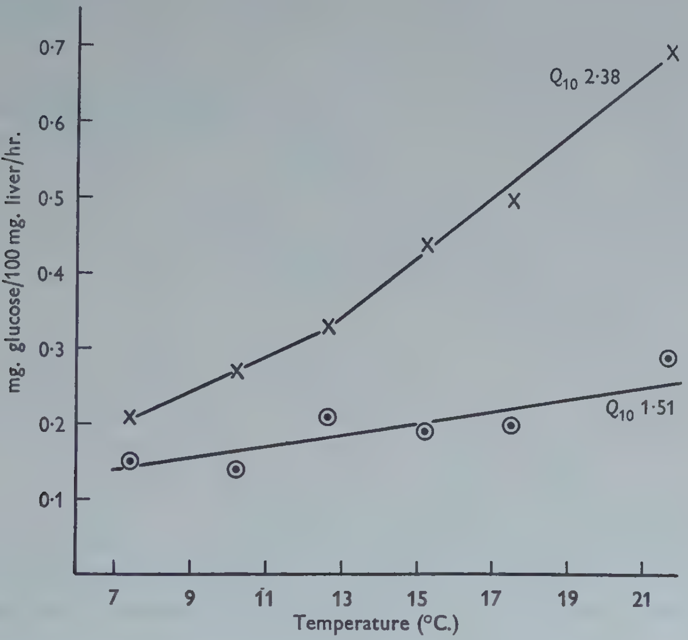

Fig. 5. Effect of dibenzyline on the glucose production of frog's liver slices, incubated *for* 1 *hr. only.* ⊙——⊙, glucose production of liver slices in Ringer plus dibenzyline (100 mg./l.). ×——×, glucose production of slices in unmodified Ringer. All slices from the same liver, and the two sets of slices immersed in their respective media at 3° C. for 30 min. immediately prior to incubation (see text). Female frog, 18 August 1953.

Environmental temperature

Kepinov (1946) found that the environmental temperature affected the rate of liver-glycogen loss in captive starved frogs. In the present work, experiments were carried out during the summer, to see if there was any relationship between the environmental temperature at which frogs were kept previous to sacrifice, and the rate of glycogenolysis in liver slices incubated over the temperature range 7–21° C.

In July, frogs were kept in the cold-room at 3° C. for periods of up to a fortnight. Control frogs were kept in the tank-room, at approximately atmospheric temperature, for the same length of time. All frogs were unfed. For each experiment, one frog from each group was killed, and liver slices taken from each animal were incubated with unmodified Ringer. The glucose production of livers from the cold-treated animals varied, and no correlation could be found with temperature. It was concluded that the rate of glucose production *in vitro* depended only on the actual temperature at which the liver slices were incubated, and not on the temperature to which the animal had previously been exposed.

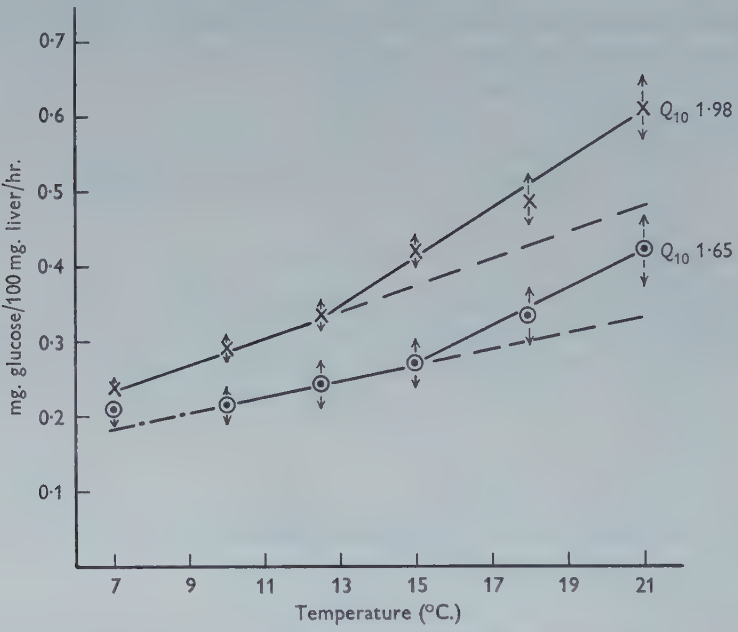

Fig. 6. Mean result of eleven experiments on the effect of dibenzyline on glucose production of frog's liver slices, incubated *for* 1 *hr. only*. ⊙——⊙, slices in Ringer plus dibenzyline (50 mg./l. in seven experiments, 100 mg./l. in four experiments, see text). ×——×, slices in control Ringer. All slices from the same liver in each experiment, and slices immersed in their respective media at 3° C. for 30 min. immediately prior to incubation. Vertical broken lines represent the standard errors of the mean. — — —, projection of the line joining the rates at 10 and 12·5° C. to 21° C.; — · —, projection of the line joining the rates at 10 and 12·5° C. after dibenzyline to 7° C.

Liver-glycogen content

The liver-glycogen content was estimated in all frogs used in this work, except in a few cases where the very small livers yielded insufficient slices for both incubation and glycogen estimation. The average liver-glycogen content of the monthly frog samples, and the pattern of seasonal change, was essentially similar to that described by Smith (1950).

DISCUSSION

The present work suggests that the low linear curve and that part of the type *B* curve below the temperature of the intersection both represent the uncomplicated action of temperature on glycogenolysis. Smith (1951) postulated that the low linear (type *E*) heart curve was the simplest relationship between pulse-rate and temperature, and it is believed that this also applies to the effect of temperature on glycogenolysis.

Supporting evidence for this hypothesis was obtained from the effect of dibenzyline added to the incubation medium. In the presence of dibenzyline there was a general tendency for type *B* curves to be changed towards the low linear form, and in one case an almost linear relation was achieved (Fig. 5). The mean curve for eleven experiments with dibenzyline (Fig. 6) shows that the extra glucose production appeared at a higher temperature and was considerably reduced in comparison with the mean control curve. If the initial linear part of the curve after dibenzyline is extrapolated to 21° C. then a line of Q_{10} 1·49 is obtained, which is almost identical with that of the mean low linear curve in Fig. 1 (1·48). The increase in mean glucose production at 18° C. could be due to adrenergic material breaking through the blockade at higher temperatures. If it may be assumed that the lower portion of the mean curve after dibenzyline (Fig. 6) represents the plain action of temperature on glycogenolysis, then that portion of the mean type *B* curve (Fig. 1) below the intersection must also represent the simple direct action of temperature, since these two lines, extrapolated to 21° C., have very similar Q_{10} values (1·49 and 1·45 respectively). The disproportionate increase in glucose production shown by the type *B* curve can only be explained by the potentiation of some glycogenolytic agent above a critical temperature.

It is known that a sympathomimetic principle does occur in the liver of the frog. Richardet (1926) found that when the medium used for perfusing a frog's liver was subsequently perfused through the isolated frog's heart, an increase in frequency and amplitude resulted. He showed that the inotropic and chronotropic effects were not due to an alteration in pH, or to the presence of glucose, and concluded that the liver had delivered a sympathomimetic substance to the perfusate. The necessary enzyme system for such a synthesis does exist in certain organs (Beyer, Blaschko, Burn & Langemann, 1950; Schmiterlow, 1951; Blaschko, 1942). Bacq (1933) and Stehle & Ellsworth (1937) put forward the theory that this liver sympathin is noradrenaline or a similar substance. Work on the comparison of the effects of noradrenaline and liver sympathin indicated that this was probably true (Greer, Pinkston, Baxter & Brannon, 1938), and Gaddum & Goodwin (1947) concluded that there is good evidence for the theory that liver sympathin is noradrenaline, or possibly tyramine. Since the present work was carried out, both noradrenaline and adrenaline have been reported in the liver of the frog (Ostlund, 1954). It would be interesting to know whether the amounts of these amines present varied with the temperature of the animal at the time of sacrifice.

The increased glucose production obtained with added adrenaline confirms the results of previous workers (Fluch, Greiner & Loewi, 1935; Kepinov, 1937), as it caused an increased glucose production at all temperatures from 7 to 21° C. Also, in the presence of adrenaline, the disproportionate increase in glucose production observed in the type *B* curves occurred at a lower temperature. Added noradrenaline also had both these effects on glucose production. This lowering of the temperature at which the increased glucose production occurred suggested that added adrenaline or noradrenaline was protecting endogenous sympathetic material from enzymic destruction, and that in the absence of either of these agents, sympathin was being destroyed at the lower temperatures and was thus unable to manifest any effect on glycogenolysis. These facts support the hypothesis that the form of the type *B* curve is due to the potentiation of liver sympathin above a critical temperature.

The results obtained with insulin both *in vivo* and *in vitro* agree with previous reports on the perfused isolated frog's liver (Issekutz & Szende, 1934). The failure of insulin to act *in vitro* is probably attributable to the time interval which elapses between administration of insulin *in vivo* and lowering of blood-sugar level (Issekutz, 1924; Smith, 1953), because when insulin is administered previously to the intact animal, the glucose production of the perfused liver is reduced (Kenese & Kovacs, 1949), as was also found with liver slices in the present work.

The general conclusion to be drawn from the experimental work presented in this paper is, therefore, that the relation between temperature and rate of glycogenolysis is fundamentally linear. Above a certain critical temperature (12–14° C.), however, it is generally found that glucose production increases more rapidly with further rise in temperature, probably owing to stimulation by endogenous sympathomimetic material. This *in vitro* relation between temperature and glycogenolysis may be considered in relation to some observations reported by Smith (1952*b*), showing that the liver glycogen content of male frogs living under natural conditions in the autumn varied inversely with temperature over the range 8–20° C. The present work, if applicable *in vivo*, suggests that glycogenolysis in the intact frog would increase rapidly with rising temperature between 13 and 21° C. It was over precisely this part of the temperature-range that Smith found the most rapid change in liver-glycogen content. This correspondence between *in vitro* and *in vivo* findings suggests that the occurrence of sympathomimetic stimulation of glycogenolysis at the higher environmental temperatures may be an important factor in controlling the glycogen content of the frog's liver in the summer and autumn months.

SUMMARY

1. The form of the temperature-glycogenolysis curve of frog's liver slices, when incubated with Ringer solution, was determined over the range 7–21° C. for samples of frogs over a period of 18 months. Three types of curve were observed:

(*a*) Low linear, interpreted as being the least complex form, in which the action of temperature is not complicated by other factors.

(*b*) Type *B*, which showed a disproportionate increase in glucose production above a certain temperature, attributed to the potentiation of sympathomimetic material.

(*c*) High linear.

2. Both the addition of adrenaline and noradrenaline to the incubation medium caused an increase in glucose production at all temperatures. Also, the dispropor-

tionate increase in glucose production of the type *B* curve occurred at a lower temperature in the presence of either of these agents.

3. Thyroxine had no effect on the form of the temperature-glycogenolysis curve, and there was no correlation between the state of activity of the thyroid gland and the type of temperature-glycogenolysis curve.

4. Glucose production of liver slices was lowered at all temperatures by the *in vivo* administration of insulin 19 hr. prior to an experiment. Insulin *in vitro* had no action.

5. Dibenzyline lowered glucose production of liver slices at all temperatures, and raised the temperature at which the extra glucose production, associated with a type *B* curve, occurred. The extrapolation of the lower portion of the mean curve after dibenzyline to 21° C. gave a line almost identical with the mean low linear curve.

6. The environmental temperature at which frogs were maintained prior to an experiment did not affect the form of the temperature-glycogenolysis curve.

7. The seasonal variation in liver-glycogen content was found to agree closely with the values obtained by Smith (1950).

I wish to thank Dr C. L. Smith for his valuable advice and criticism during the course of this work. I should also like to thank Dr K. Carter, of Smith, Kline and French Ltd., for the sample of dibenzyline given to Dr Smith.

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HORMONAL CONTROL OF OVARY DEVELOPMENT IN MOSQUITOES

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The control of ovary development by hormones has been shown in a number of insects, and evidence has been brought forward to demonstrate it in mosquitoes. It has been claimed on experimental grounds that a gonadotrophic hormone is secreted by the corpora allata after the intake of blood by female mosquitoes, co-ordinating ovary development with the supply of food (Detinova, 1945), and histological evidence has been produced connecting the corpora allata with ovary development (Mednikova, 1952).

Culex pipiens L. contains races which differ in their requirements for ovary development. One race is autogenous, not requiring any food during the adult stage for ovary development to occur, another race is anautogenous and the ovaries will not develop beyond a resting stage until a blood meal of adequate size has been taken. These differences must reflect differences in hormone secretion if the development of the ovaries is controlled by hormones. These and other mosquitoes have therefore been examined for evidence of humoral control of ovary development and for differences between the autogenous and anautogenous forms.

From an abundance of work it is clear that autogenous and anautogenous strains of *C. pipiens* which are genetically distinct occur in nature (Roubaud, 1930; Callot, 1947), while some strains contain both forms in varying proportions (Knight & Abdel Malek, 1951). Anautogeny seems to show incomplete dominance over autogeny (Buck, 1935; Tate & Vincent, 1936) and a unifactorial basis for the character has been questioned (Kitzmiller, 1953). The only previous explanation of the mechanism of autogeny stated that the two forms differed in the extent of the reserves carried over to the adult stage; it was considered that autogenous mosquitoes carried over sufficient reserves to develop the ovaries while anautogenous mosquitoes did not (Boissezon, 1933; Roubaud, 1933). Autogeny is known in *Drosophila*, and a similar explanation has been put forward to account for the different food requirements of *D. melanogaster* and *D. virilis* (Bodenstein, 1947).

The account which follows describes the reserves of autogenous and anautogenous mosquitoes, the evidence from histology for hormone secretion and experimental evidence for humoral control of ovary development.

MATERIAL

Culex pipiens pipiens L. Anautogenous, eurygamous (i.e. requiring a large space for copulation), bird-biting and diapausing. Cultures were maintained by collecting in Cambridge wild female mosquitoes which had already fed and mated.

Culex pipiens form *molestus* Forskål. Autogenous, stenogamous (i.e. able to copulate in a small space), man-biting and non-diapausing. This strain, which had been cultured in the laboratory for many years, was obtained from Elberfeld in Germany in 1933.

Culex pipiens form *berbericus* Roubaud. Largely anautogenous but with a very low percentage of autogeny, stenogamous, man-biting and non-diapausing. The culture was developed from eggs obtained in the Jardin d'Essai in Algiers. It was selected for anautogeny and was used as an anautogenous strain in blood-feeding experiments in which *C. pipiens pipiens* would not feed readily.

Experiments were also performed on females of *Aëdes aegypti* (L.), *Anopheles stephensi* Liston and *A. labranchiae atroparvas* van Thiel. This last species cannot be positively identified with the *A. maculipennis* sensu lato used by Detinova (1945), but it must at least be closely related to it.

THE NATURE AND EXTENT OF THE RESERVES

Although autogenous mosquitoes are considered to differ from anautogenous mosquitoes principally in the extent of the reserves carried over to the adult stage, no detailed comparison has been made of the reserves of the two forms beyond the assertion that the larvae of autogenous strains contain reserves at least three times as extensive as those of the larvae of anautogenous strains cultured under similar conditions (Roubaud & Toumanoff, 1930; Roubaud, 1933). However, poor larval nutrition has been shown to affect only the number of eggs produced by autogenous females and not the capacity to produce eggs without feeding (Gaschen, 1932; Hecht, 1933); even larvae reared at the starvation limit produced females which were all autogenous (Buck, 1935).

It is known that the fat-body cells of the larva of *Culex pipiens* contain droplets of fat, protein and glycogen (Boissezon, 1930, 1932), and a similar structure has been described in the larva of *Aëdes aegypti* (Wigglesworth, 1942). The massive abdominal muscles of the larva of *Culex pipiens* are carried over intact to the adult stage when they are autolysed, apparently providing additional protein for ovary development in the autogenous form and extending the fat-body reserves of hibernating anautogenous females (Roubaud, 1932, 1933).

Methods

Larvae of all strains were cultured in the suspensions of bacteria which develop over ground dog biscuit in water and were provided with a slight excess of food to ensure adequate nutrition. Larvae about to pupate, in which the respiratory horns had become pigmented, were chosen for examination, and the methods for staining fat-body cells described by Wigglesworth (1942) were used. To stain glycogen, specimens were fixed in Carnoy's fluid, stained in a mixture of absolute alcohol saturated with iodine (65 parts) and 1 % iodine in potassium iodide (35 parts), dehydrated in 95 % alcohol saturated with iodine and mounted in euparal. Fat and protein droplets were stained in the same specimen by fixing in Baker's formaldehyde calcium and staining first with Sudan IV and then with ninhydrin.

The reserves of autogenous and anautogenous Culex pipiens

Dissection of fully grown fourth-instar larvae showed that the fat-body lobes of *Culex pipiens pipiens* were rather less extensive than those of *C. pipiens* form *molestus*, but that they were developed to a sufficient extent to contain a considerable amount of reserves. The abdominal muscles were extensively developed in both forms. No differences were found in the nature or extent of the reserves within the fat-body cells. Before pupation the cells were packed with large droplets of fat and smaller droplets of protein and glycogen. The difference in the extent of the fat body appeared slightly greater in the adult, but it was clear that the anautogenous *C. pipiens pipiens* contained sufficient reserves to develop a number of eggs, for the fat body contained considerable quantities of protein and the abdominal muscles provided much more.

This study of the fat body led to interesting observations on its coloration. In well-nourished cultures the fat body of the fully grown larva was often strongly pigmented, green in *C. pipiens* form *molestus* and pink or purple in *C. pipiens* form *berbericus*. The coloration was found to be confined to the protein droplets, and when Gmelin's reagent was added to exposed fat-body cells the pink colour turned to green and then to blue, while the green-coloured fat body developed a bluish colour. This suggested that the colours were due to bile pigments. The fat body was pigmented in the adult mosquitoes at emergence, but within a day the pigment disappeared from the fat body and accumulated in the pericardial cells. Strains of *C. pipiens* have been described with the larval fat body coloured green and brown, the colour forms being inherited in simple Mendelian ratios when the strains were crossed (Huff, 1929).

The mobilization of the reserves

The fate of the reserves contained in the fat body and the abdominal muscles during the first 5 days of adult life is shown in Table 1, together with the state of the ovaries. The extent of the fat body is expressed by the numbers 5 (extensive) to 1 (very slight), that of the abdominal muscles by the numbers 4 to 0. The categories of ovary development described by Christophers, Sinton & Covell (1936) are used to show the state of the ovaries. The ovaries of anautogenous mosquitoes enter a resting stage during stage II of Christophers *et al.*, and further growth is dependent on blood feeding. For convenience, stage II has been divided into stage II*a* (ovaries entering or in the resting stage) and stage II*b* (ovaries which have passed the resting stage but which have not reached stage III).

The ovaries of *C. pipiens* form *molestus* were seen to develop to maturity within 5 days; concurrently the fat body became greatly reduced and the abdominal muscles were autolysed, disappearing completely by the second day after emergence (Table 1). In *C. pipiens pipiens* the ovary did not develop beyond the resting stage in the absence of a blood meal, but both fat-body and muscle reserves were mobilized. The apparent increase of the fat body after emergence was probably due to the variability of the material. It may be noted that decapitation and ligation of the abdomen of *C. pipiens* form *molestus* within an hour of emergence did not prevent the utilization of the fat-body reserves or the breakdown of the abdominal muscles.

Table 1. *The state of the ovaries, fat body and abdominal muscles of females of autogenous and anautogenous forms of* Culex pipiens *under various conditions. Each daily entry is the record of five individuals and shows the range of observations on ovary development and the mean of the observations on fat body and muscle*

Species	Organ	Days after emergence					
		0	1	2	3	4	5
C. pipiens form *molestus* unfed	Ovary	I	II*a*	II*b*–III	III	IV	V
	Fat body	5	4·8	4·2	2·1	1	1
	Muscles	4	2·8	0	0	0	0
C. pipiens pipiens unfed	Ovary	I	I	I	I–II*a*	I	I–II*a*
	Fat body	2·8	4	3·6	1·8	1·4	1
	Muscles	4	2	0	0	0	0
C. pipiens form *molestus* decapitated within 1 hr. of emergence	Ovary	—	I	I–II*a*	I–II*a*	I–II*a*	I–II*a*
	Fat body	—	4·2	3·4	2·2	2·2	1·8
	Muscles	—	2·4	0·2	0	0	0
C. pipiens form *molestus* abdomen ligated within 1 hr. of emergence	Ovary	—	I	I	I–(II*b*)*	I	I–II*a*
	Fat body	—	3·6	3·4	3·2	2·6	2·2
	Muscles	—	1·2	0·8	0	0	0
Species	Organ	Days after blood meal					
		0	1	2	3	4	5
C. pipiens form *berbericus* fed human blood	Ovary	—	II*b*–III	III	III–V	III–V	V
	Fat body	—	1·8	1·8	1·6	1	1
	Muscles	—	0	0	0	0	0

* One specimen had two follicles at stage II*b* and its remaining follicles at stages I or II*a*.

Staining the inclusions of the fat-body cells showed that the various reserves were not all used concurrently. The 48-hour-old female of *C. pipiens* form *molestus* had abundant fat, glycogen and protein in its fat-body cells, at 72 hr. there was very little protein but much fat and glycogen, and at 120 hr., when the eggs were mature, there was no protein and only traces of fat and glycogen. Females of this age, in which ovary development had been prevented by ligation, contained some fat and glycogen and abundant protein. The fat-body cells of females of *C. pipiens pipiens* 96 hr. after emergence contained small amounts of fat and glycogen and appreciable amounts of protein. After 120 hr. there were only traces of fat and glycogen and no protein.

THE HISTOLOGY OF THE ENDOCRINE ORGANS

Little is known of the structure and function of the endocrine organs of mosquitoes. Their structure has been described in part (Bodenstein, 1945; Cazal, 1948) and the corpora allata have been linked with ovary development on histological grounds. Detinova (1945) found that the size of the corpora allata of *Anopheles maculipennis* sensu lato rapidly decreased after the intake of blood, and Mednikova (1952) deduced from a histological study that the corpora allata stimulated ovary development in *Anopheles* and *Chaoborus*. In the present work, female pupae and adults of *Culex pipiens* were fixed in aqueous Bouin and stained with Gomori's chrome haematoxylin. Other stains used were Masson's trichrome stain and Samuel's silver stain.

Neurosecretory cells

Neurosecretory cells were found in the supra- and suboesophageal ganglia and in the thoracic and abdominal ganglia in pupae and adults of *Culex pipiens*. In *Calliphora*, the neurosecretory cells of the supraoesophageal ganglion have been shown to play an important part in ovary development (Thomsen, 1952), and particular attention was paid to these cells in *Culex pipiens*. A group of about ten neurosecretory cells occurred in the pars intercerebralis, and others were to be found in different parts of the supraoesophageal ganglion. The cytoplasm of the cells contained numerous granules which stained blue with Gomori's chrome haematoxylin, but the axons of the cells could be traced only a short distance and no neurosecretory granules could be seen in them. The neurosecretory cells were closely examined for signs of secretion in *C. pipiens* form *molestus* during the 24 hr. after emergence, in which period experiments showed ovary development was stimulated, and in *C. pipiens* form *berbericus* during the 24 hr. following blood feeding. However, no changes in the granular content of the cytoplasm could be seen.

Peritracheal glands and corpora allata

The peritracheal glands of *C. pipiens*, which surround the corpora allata, degenerate during the late pupal stage and are unlikely to play any part in ovary development.

The corpora allata were closely examined for secretory activity in females of *C. pipiens* form *molestus* during the 24 hr. after emergence and in *C. pipiens* form *berbericus* during the 24 hr. after feeding on blood. No change in the volume of the corpora allata was apparent over these periods. In certain individuals, the corpora allata had a vacuolate appearance and the vacuoles occasionally contained chromatic droplets stained black by Gomori's chrome haematoxylin (Fig. 1 *a*), but the random occurrence of this condition suggested that it was not connected with the development of the ovaries.

Corpora cardiaca

Cazal (1948) described an unpaired corpus cardiacum, in the pupa of *C. pipiens*, fused with the hypocerebral ganglion and lying below the aorta in the neck. Examination of the hypocerebral ganglion, in pupae stained with Gomori's chrome haematoxylin after fixation in aqueous and alcoholic Bouin, showed a group of cells, similar to brain cells, lying below the aorta in the neck region and receiving the recurrent nerve and the two cardiacal nerves from the brain. Cells were found, close to the hypocerebral ganglion, which resembled those figured by Cazal as part of the corpus cardiacum. These cells could be seen surrounding the pharynx in this region, apparently forming part of the wall of the pharynx. In the pupa there is a group of these cells just below the hypocerebral ganglion, but in the adult, not examined by Cazal, they become flattened into a single layer.

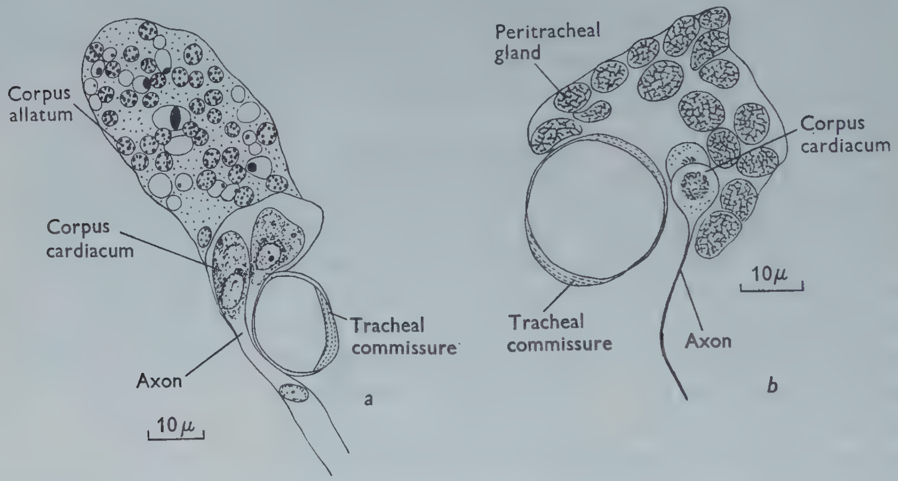

Fig. 1 *a*, *b*. Endocrine organs of *Culex pipiens*. *a*. Sagittal section of the corpus allatum and corpus cardiacum of an adult female of *C. pipiens* form *berbericus*, stained with Gomori's chrome haematoxylin. *b*. Sagittal section of the peritracheal gland and corpus cardiacum of a female pupa of *C. pipiens* form *molestus*, stained with Samuel's silver stain.

Two groups of cells, previously undescribed, are to be found between the corpora allata and the tracheal commissure (Fig. 1 *a*). Each group consists of three or four large cells with large nuclei and extensive cytoplasm containing granular material which stains with Gomori's chrome haematoxylin. From these cells axons run forward under the tracheal commissure in the cardiacal nerve. The axons, consistently found in Gomori-stained sections, were also found in pupal material stained with Samuel's silver stain after fixation in the recommended modification of Bouin (Samuel, 1953) (Fig. 1 *b*). In the late fourth-instar larva, some cells with extensive, clear cytoplasm, which probably corresponded with the newly described cells found in the pupa and adult, were occasionally seen between the peritracheal gland cells near the tracheal commissure. The position of these two groups of cells and their paired condition suggest that they comprise the corpora cardiaca.* Axon-like structures, running from the cells of the corpora cardiaca into the cardiacal nerves, have been described in the larva of *Ptychoptera* (Thomsen, 1951).

No evidence was obtained to suggest that the corpora cardiaca were active in ovary development.

* That the bodies are cells and not swollen nerve endings has been confirmed by phase contrast examination of living material. The 'nerve' appears to be a homogeneous protoplasmic trunk surrounded by a very fine sheath.

EXPERIMENTS ON OVARY DEVELOPMENT

Detinova (1945) claimed that ligation of *Anopheles maculipennis* sensu lato after a blood meal did not prevent ovary development if the ligature was tied between the head and thorax, but did prevent ovary development if it was tied between the pro- and mesothorax within 6 hr. of feeding. From this result she concluded that the corpora allata stimulated ovary growth by secreting a gonadotrophic hormone after blood feeding. Similar experiments have been performed on the autogenous mosquito, *Culex pipiens* form *molestus*, after emergence of the adults, and on the following anautogenous mosquitoes after blood-feeding, *C. pipiens* form *berbericus*, *Aëdes aegypti*, *Anopheles labranchiae atroparvus* and *A. stephensi*. The strain of *Culex pipiens* form *berbericus*, which was largely anautogenous and selected for anautogeny, was used instead of the entirely anautogenous *C. pipiens pipiens* because the latter is very difficult to feed under experimental conditions. Mosquitoes were decapitated or were ligated at the base of the abdomen using a fine hair. Decapitation removes the neurosecretory cells of the brain and severs the cardiacal and recurrent nerves, while ligation isolates the ovaries from all the endocrine organs outside the abdomen. In the later ligation experiments, those performed on *Anopheles stephensi*, *A. labranchiae atroparvus* and *Aëdes aegypti*, the head and the front of the thorax were cut away after the ligature had been tied, but no change in result was observed. The ovaries of the decapitated mosquitoes were examined 5 days after operation, and those of ligated specimens, which survived less well, after 3 days.

Results

Females of *Culex pipiens* form *molestus* were decapitated or ligated at the base of the abdomen at hourly intervals after emergence from the pupa, and the subsequent ovary development of these mosquitoes was studied (Figs. 2, 3). No specimens decapitated earlier than 7 hr. after emergence developed their ovaries beyond the resting stage, while many of those decapitated more than 7 hr. after emergence developed their eggs to maturity or to a lesser degree. Among the ligated specimens, none ligated earlier than 5 hr. after emergence developed its ovaries beyond the resting stage, but many of those operated on more than 5 hr. after emergence did so.

When the anautogenous females of *C. pipiens* form *berbericus* were decapitated immediately after feeding on human blood, a proportion of them developed their ovaries beyond the resting stage. This was in accordance with the result obtained by Detinova (1945) in similar experiments on *Anopheles maculipennis* sensu lato. The result obtained by ligating the abdomens of females of *Culex pipiens* form *berbericus* after blood feeding was quite different from that obtained by Detinova. Females ligated as soon as 3–4 min. after the *start* of blood feeding developed their ovaries beyond the resting stage (Fig. 4).

Ligation experiments were also performed on other anautogenous species of mosquito after feeding on human blood. Females of *Aëdes aegypti* which had been ligated as early as 2–3 min. after the start of feeding developed their ovaries beyond the resting stage (Fig. 5), and females of *Anopheles labranchiae atroparvus* ligated 1–2 min. after starting to feed developed their ovaries beyond the resting stage (Fig. 6).

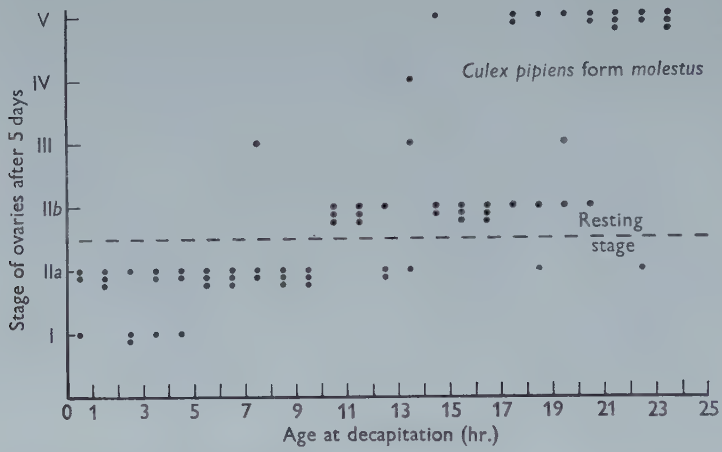

Fig. 2. The effect of decapitation on the development of the ovaries of *Culex pipiens* form *molestus* when performed at hourly intervals after emergence. Each point represents one female.

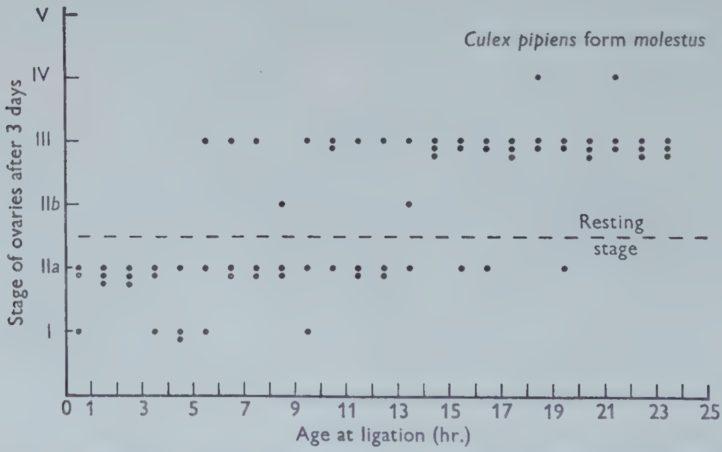

Fig. 3. The effect of ligation on the development of the ovaries of *Culex pipiens* form *molestus* when performed at hourly intervals after emergence. Each point represents one female.

A. stephensi was the only anautogenous mosquito which gave results similar to those described by Detinova for *A. maculipennis* sensu lato. Fifty specimens (of which only a few are shown in Fig. 7) ligated within 1 hr. of feeding did not develop their ovaries beyond the resting stage. Among those ligated 2 or more hours after feeding occasional specimens developed their ovaries to stage II *b*, and among females ligated 9 hr. after feeding a considerable proportion reached this stage. From 18 hr. onwards a number of specimens developed their eggs to maturity.

Implantation experiments

The anautogenous females of *Culex pipiens pipiens* had been shown to contain sufficient reserves for the development of a number of eggs without the addition of protein from ingested blood, and an attempt was made to stimulate the development of the ovaries of unfed females by implanting active endocrine organs. In early experiments brains, corpora allata and corpora cardiaca, removed from females of *C. pipiens* form *molestus* a few hours after emergence, were transplanted into the abdomens of females of *C. pipiens pipiens*. No positive results of ovary development were obtained, but the difficulty of handling the small endocrine organs suggested that the transplantations were not successfully performed.

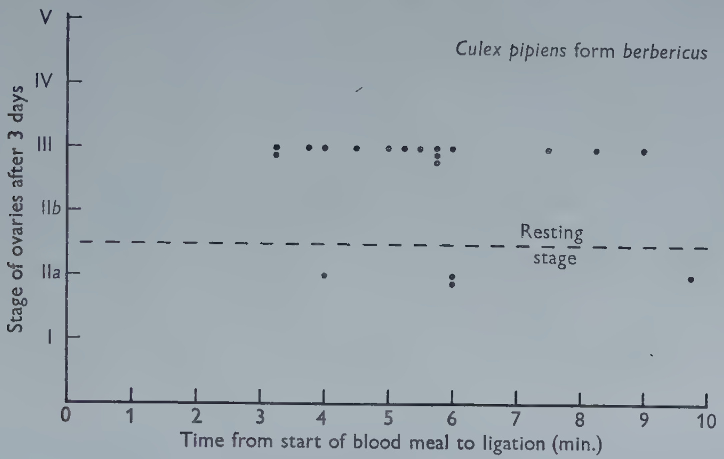

Fig. 4. The effect of ligation on the development of the ovaries of *Culex pipiens* form *berbericus* when performed immediately after blood feeding. Each point represents one female.

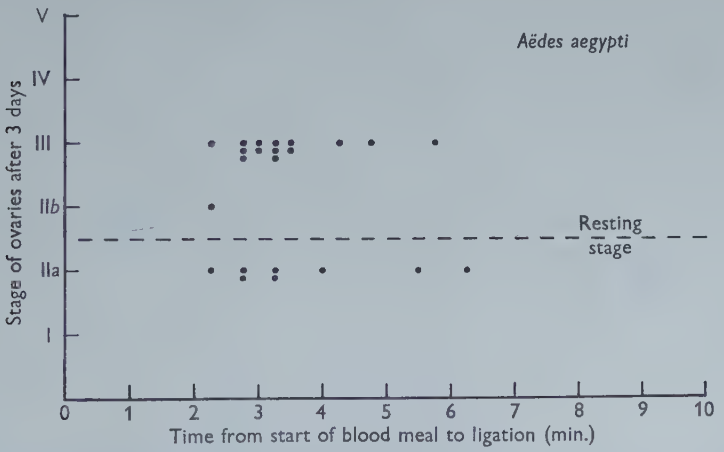

Fig. 5. The effect of ligation on the development of the ovaries of *Aëdes aegypti* when performed immediately after blood feeding. Each point represents one female.

Thomsen (1952) prevented ovary development in *Calliphora erythrocephala* by removing the median neurosecretory cells from young females, and was able to restore ovary development in a small proportion of the operated flies by transplanting, into each, three corpora allata-cardiaca systems from female flies fed meat for 7 days. Similar transplantations were made into sugar-fed females of *Culex pipiens pipiens*, three corpora allata-cardiaca systems from females of *Calliphora* fed meat for 6–7 days being implanted into the abdomen of each mosquito. However, in twenty-seven individuals into which the endocrine organs had been successfully implanted and which lived for 5 days after the operation, no development of the ovaries beyond the resting stage was found.

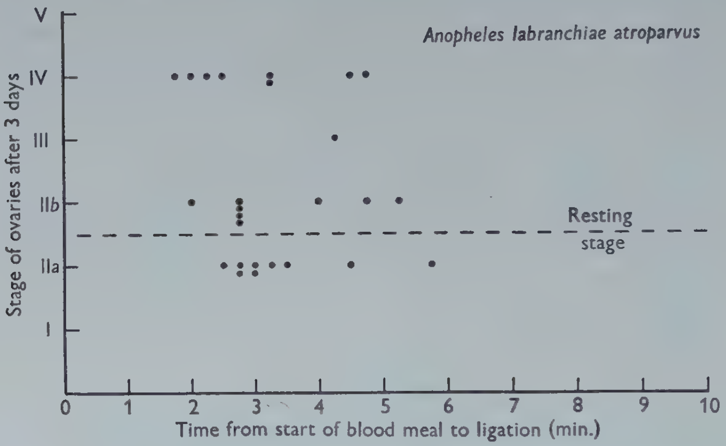

Fig. 6. The effect of ligation on the development of the ovaries of *Anopheles labranchiae atroparvus* when performed immediately after blood feeding. Each point represents one female.

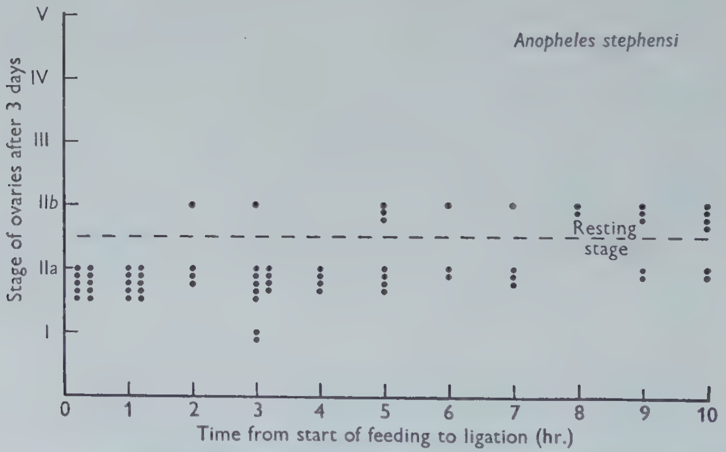

Fig. 7. The effect of ligation on the development of the ovaries of *Anopheles stephensi* when performed at various times after blood feeding. Each point represents one female.

DISCUSSION

Autogeny in *Culex pipiens* form *molestus* has been said to result from the accumulation of additional reserves during the larval stage. As the anautogenous form, *C. pipiens pipiens*, has now been shown to contain considerable reserves, some other explanation of autogeny must be looked for. Both decapitation of *C. pipiens* form *molestus* and ligation at the base of the abdomen, when performed within a few hours of emergence, prevented the development of the ovaries which would normally have taken place. The simplest explanation of this result is that the operation prevents the working of a gonadotrophic hormone. This explanation is not supported by additional evidence from histology or from implantation experiments, but it is not inconsistent with what is already known of insect hormones. Decapitation removes the neurosecretory cells and cuts the cardiacal nerves and the recurrent nerve, while ligation isolates the ovaries from all endocrine organs outside the abdomen. There is thus sufficient experimental evidence to suggest that the development of the ovaries in autogenous mosquitoes is stimulated by a gonadotrophic hormone secreted within a few hours of emergence, presumably by the corpora allata.

Detinova (1945) claimed that in *Anopheles maculipennis* sensu lato ovary development was stimulated by the secretion of a gonadotrophic hormone by the corpora allata some hours after feeding on blood, and that ovary development could be prevented by ligating behind the corpora allata. In three species of mosquito ligation failed to prevent ovary development when it was performed immediately after feeding, in some cases 2–3 min. after the start of feeding. Only in *A. stephensi* did there appear to be a critical period, the ovaries developing only when the ligature was tied 2 hr. or more after feeding. If the critical period in *A. stephensi* represents a period of hormone secretion, then the situation appears only slightly different from that in *Culex pipiens* form *molestus*, the blood meal stimulating secretion of the gonadotrophic hormone instead of secretion occurring automatically after emergence.

On the present evidence, no straightforward theory of hormone secretion can be applied to the other species, *Culex pipiens* form *berbericus*, *Aëdes aegypti* and *Anopheles labranchiae atroparvus*. It seems unlikely that, where ovary development takes place despite ligation within a very few minutes of the start of feeding, sufficient hormone can pass into the abdomen. If the hormones are secreted before feeding takes place, some chemical factor in the food or some behavioural stimulus from feeding may trigger ovary growth. A chemical factor in the blood has been sought by a number of workers, but there is little evidence to suggest that more than protein is required (Yoeli & Mer, 1938; Greenberg, 1951; Hosoi, 1954). Clearly, further evidence is necessary for an understanding of the control of ovary development in these anautogenous mosquitoes.

SUMMARY

A study was made of ovary development in *Culex pipiens* form *molestus* Forskål, an autogenous mosquito not needing food in the adult stage to develop its eggs, and in *C. pipiens pipiens* L. and other anautogenous mosquitoes which require blood for ovary development.

Comparison of the reserves of fully grown larvae of the autogenous and anautogenous forms of *C. pipiens* showed that the autogenous form had a rather larger fat body, but that the anautogenous form contained sufficient protein reserves to develop a number of eggs. It was considered that autogeny did not depend solely upon the ability to amass extensive reserves but also upon some other physiological mechanism.

Decapitation and ligation at the base of the abdomen prevented ovary development in *C. pipiens* form *molestus* when performed within a few hours of emergence, but when performed 7 or more hours after emergence it often failed to prevent ovary development. It is suggested that a gonadotrophic hormone is secreted during this time.

Ligation of the abdomen within an hour of feeding on blood appeared to prevent ovary development in *Anopheles stephensi* Liston. Ovary development occurred in a small proportion of females ligated 2 or more hours after feeding, and this proportion increased with time.

Ligation of the abdomen immediately after blood feeding failed to prevent ovary development in *Culex pipiens* form *berbericus* Roubaud, *Aëdes aegypti* (L.) and *Anopheles labranchiae atroparvus* van Thiel, even in some cases where the ligature was tied within 2–3 min. of the start of feeding.

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FACTORS CONTROLLING THE DIURNAL RHYTHM OF ACTIVITY OF *PERIPLANETA AMERICANA* L.

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(*Received 7 October* 1955)

INTRODUCTION

Two main lines of approach are evident in recent work on diurnal rhythms of activity in animals. On the one hand investigators have been determining the form of the activity rhythm of many animals, particularly insects, and the effects of various environmental factors on these rhythms. On the other a small amount of work has been carried out on the internal factors which are involved in the mechanisms of the rhythm. From the results of the former line of investigation it appears that light or darkness is the factor which fixes the rhythm in most cases, while temperature may modify but not impress a rhythm. The most significant feature of these results is that in most animals it has appeared that once an activity rhythm is established by means of alternating light and darkness then that rhythm continues for a period of time under new environmental conditions. This implies the presence of an 'internal clock' which is regulated by the external conditions but can function without regulation for a period varying in different animals from a few days, as in cockroaches, to months or longer, as in some beetles (Park, 1932) and mayflies (Harker, 1953).

A possible 'internal clock' in *Periplaneta americana* L. is described in the following pages, and the way in which it may be regulated by the environment is discussed.

METHODS

The activity of *P. americana* was measured using two different methods. In one an actograph was used, consisting of an oblong box 8 × 4 in. which was balanced on a knife-edge and had a central wire which brushed through a mercury switch so that an electric circuit was closed whenever the actograph tipped. Recordings were made on a smoked drum. The main disadvantage of this method is that only some of the movements of the cockroach are recorded, either movements along the whole length of the box or smaller movements about the central region. Also the cockroach cannot be given much food or water at a time because the balance of the box is upset as it is consumed. Feeding itself is an activity which is not often recorded by an actograph as the animals may only be moving about in a very limited area. The advantage of the method lies in the fact that the movements of the cockroach are perhaps not as likely to be affected by extraneous factors as in other methods.

The second method of recording activity was to attach a fine thread to the pronotum by a tiny drop of wax, the other end of the thread being attached to a light glass capillary tube acting as a lever. The free end of the tube recorded its movements on a smoked drum. This method recorded all the movements of the cockroach, however small, and did not appear to disturb the animal. Food and water could be left in the cage with the animal in large quantities. In this method the disadvantage lay in the difficulty of analysing the smoked drum record. Under normal environmental conditions the period of activity is a definite one and can be seen at a glance, but when the animals are being subjected to experimental conditions some method of assessing the number of movements is essential. The method adopted was to count only vertical marks of 5 mm. and over, and to count any vertical mark as one movement however great its amplitude. Without knowing the direction of a movement the amplitude could not be taken as a measure of the distance moved by the cockroach. In any case no greater accuracy was required than was given by this means. The second method was used for all experiments and a small number of trials for each was run using the actograph as a check.

All experiments except those carried out in natural daylight and darkness were conducted in a light-tight chamber in which the light was provided by a 60 c.p. lamp giving a light of 25 f.c. at the position of the cockroach. The light periods were controlled by a time switch. Temperature was not controlled but was normal room temperature, preliminary experiments having shown that temperature only affected the amount and not the rhythm of activity (cf. Cloudsley-Thompson, 1953). All experiments were repeated at least five times. Only adult males were used for the experiments in case females might be affected by a reproductive cycle.

ENVIRONMENTAL FACTORS AND THE RHYTHM OF ACTIVITY

(*a*) *Alternating light and darkness*

Under conditions of alternating light and darkness *P. americana* exhibits a remarkably constant type of activity rhythm. The few insects which showed any different rhythm were all found to have some major injury or died within a few days.

There is a substantial increase in activity at the beginning of the dark period, and this reaches its peak, on an average, in 2 hr. The peak is only sustained for from 30 min. to 1 hr. and gradually falls off over the following 2 hr. The animals move about very little during the remaining hours of darkness or in the light period, but after about 6 hr. of light activity begins to increase slightly (Fig. 1 *a*). The same activity curve is obtained whether the animals are kept in natural daylight and darkness or in artificial light and darkness. The activity of over a hundred cockroaches has at various times been recorded under these conditions.

(*b*) *Reversed light and darkness*

When cockroaches which have previously been in alternating daylight and darkness are transferred to conditions in which the light periods are altered, for example, the times of light and darkness entirely reversed, the old rhythm of activity continues for from 2 to 4 days, but is gradually lost and replaced by a new rhythm with again the peak of activity occurring in the dark period.

A new rhythm can be induced provided that neither the dark nor the light period is of less than 2 hr. duration.

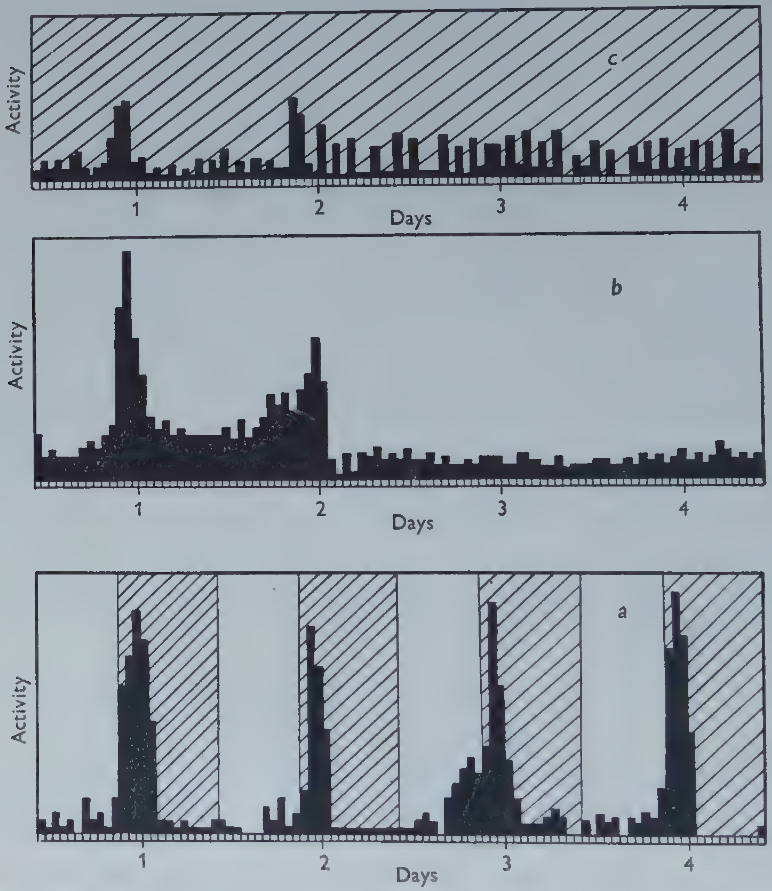

Fig. 1. The activity of *P. americana* at room temperature (*a*) in alternating light and darkness, (*b*) in continuous light, (*c*) in continuous darkness. Hatched areas represent dark periods.

(*c*) *Continuous light or darkness*

When cockroaches are transferred from alternating light and darkness into continuous light or continuous darkness the original rhythm of activity is carried on over a period of 3–5 days, but becomes gradually less marked during that period. After the fifth or sixth day in constant light activity becomes random, but total activity per day remains the same as under the previous conditions. Activity tends to be low but fairly constant (Fig. 1*b*).

In continuous darkness activity is lower on the first day than it is on the first day in continuous light. For example, in one set of experiments there was an average of 79 movements compared with 186. After the fifth or sixth day in continuous darkness the total activity per day remains the same as under the previous conditions but activity is spasmodic, very high and then very low, and the animals are seldom active in two successive hours (Fig. 1*c*).

When cockroaches are subjected to alternating light and darkness, after having been in continuous light or darkness, they pick up a new rhythm of activity immediately without any initial conditioning period.

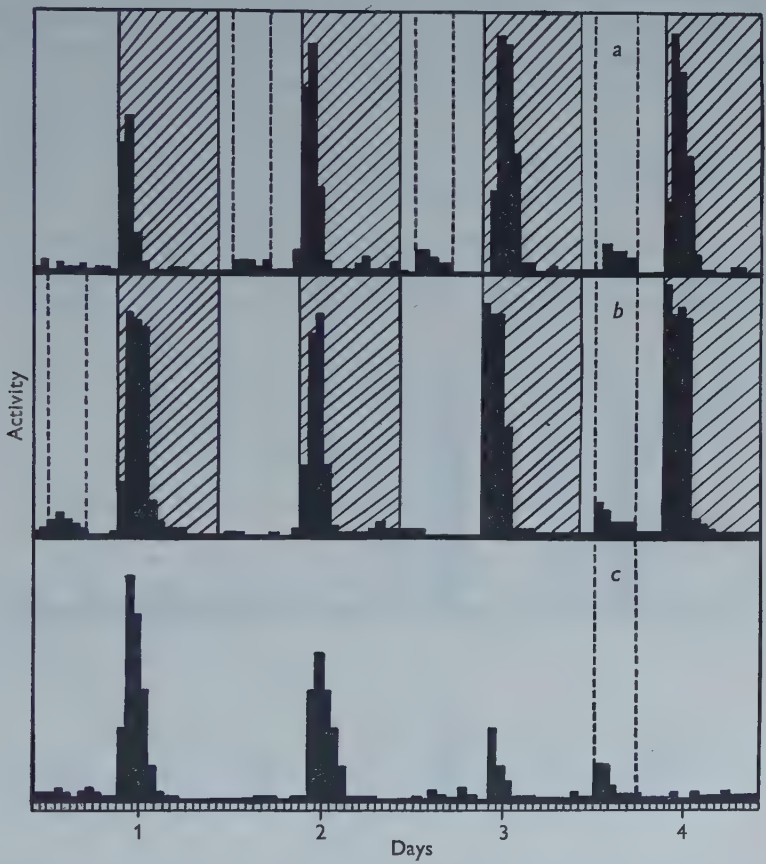

Fig. 2. (*a*) The activity of *P. americana* in alternating light and darkness when food is obtainable only in the light period. The cockroaches had been starved for 4 days previous to the experiment. (*b*) The activity after 3 weeks of above conditions when food was not given at the usual time. (*c*) Activity after food had been introduced at usual time for 5 days, and then animals transferred to continuous light and starved. Period when food present shown between dotted lines. Dark period hatched.

(*d*) *Feeding in relation to activity*

During the active period while the animal is in darkness most of the feeding takes place. The relationship between the hunger cycle and the rhythm of activity was investigated as follows. Cockroaches were starved for 4 days and then food and water were placed in the cage from 12 noon to 5 p.m. when it was removed. This was continued for a period of 3 weeks, the animals meanwhile being in alternating light and darkness, the dark period of which lasted from 8 p.m. to 5 a.m. The animals showed a normal activity rhythm throughout with the peak of activity occurring during the dark period. They showed a slight increase in activity when the food was in the cage, but when the food was not introduced at the usual time there was no increase in activity. Therefore even if it appeared that a rhythm was formed it was not a true one which could be carried over under new environmental conditions, as was shown when the cockroaches were moved to constant light conditions (Fig. 2).

It follows from these experiments that the activity rhythm is not likely to be the expression—at least, not the direct expression—of a hunger cycle.

INTERNAL FACTORS AND THE RHYTHM OF ACTIVITY

(*a*) *Parabiotic experiments*

Cockroaches were joined together in parabiosis following the method used by Bodenstein (1953), the notum being the point of contact. The legs were removed from the uppermost cockroach to prevent it clinging to the side of its cage or its food.

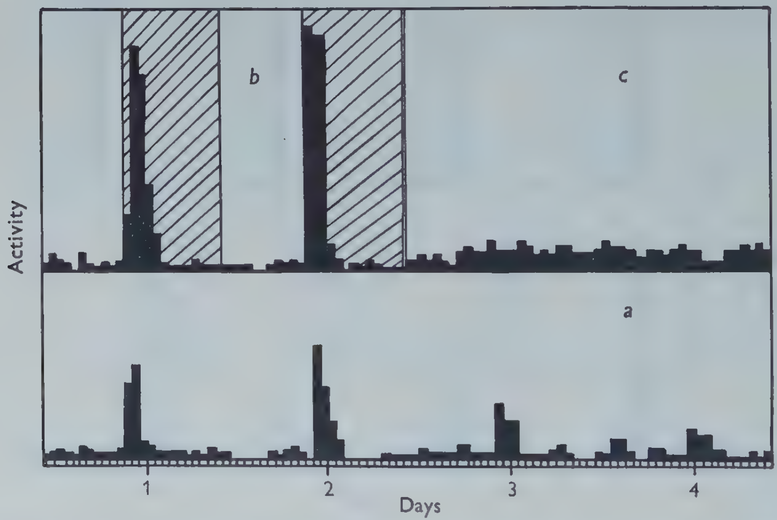

Fig. 3. (*a*) The activity of a parabiotic pair of cockroaches in continuous light. The lower, mobile cockroach had previously been in continuous light and had shown the arrhythmic activity recorded in (*c*), the upper cockroach had previously been in alternating light and darkness and had shown the rhythm of activity recorded in (*b*). Dark periods hatched.

A cockroach which had previously been in alternating light and darkness, and had a normal rhythm of activity, was joined in parabiosis with another which had been in constant light for the whole of its life, and showed no rhythm of activity. The arrhythmic cockroach was the lower and mobile one of the pair. The pair were left in constant light and their activity measured. They were found to show a rhythm similar to that previously shown by the uppermost, immobilized cockroach (Fig. 3).

If left in constant light this rhythm was gradually lost, as is that of any cockroach in constant light.

If two cockroaches were joined together in the reverse position, the mobile one being that from conditions of alternating light and darkness, then the pair still showed a normal rhythm of activity.

Painting over the eyes and ocelli has been found to cause a loss of rhythm (Cloudsley-Thompson, 1953). Two cockroaches which had previously been kept in constant light and were arrhythmic were joined together in parabiosis, the eyes and ocelli of the mobile one were painted over and the pair placed in alternating light and darkness. A normal rhythm of activity developed (Fig. 4). Controls were tested in which both cockroaches of the pair had had the eyes and ocelli blackened, in this case no rhythm was developed.

It would appear from this set of experiments that a secretion, carried in either the blood or tissues, is involved in the production of the diurnal rhythm.

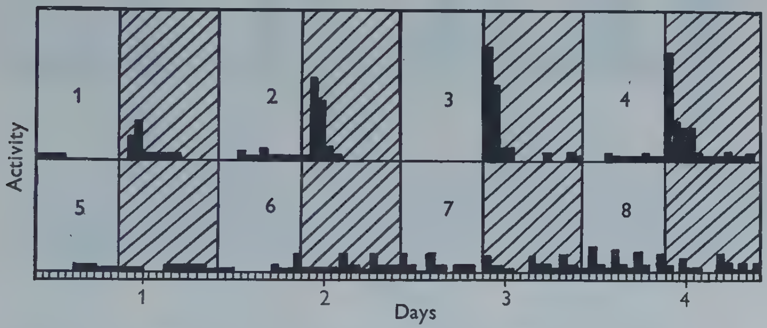

Fig. 4. The activity of a parabiotic pair of cockroaches in alternating light and darkness. Both had previously been in continuous light and the eyes and ocelli of the lower, mobile one, were blackened.

(*b*) *Neurosecretory organs in relation to the activity rhythm*

P. americana will go on moving about quite actively for up to 10 days after decapitation. The activity of headless cockroaches has been measured and found to show no rhythm (Fig. 5*a*).

Pairs of sub-oesophageal ganglia taken from cockroaches with normal activity rhythms were implanted, one each, into headless cockroaches. The implant was made in the abdomen just lateral to the dorsal heart. The implanted cockroaches were placed in continuous light. Their subsequent activity is shown in Fig. 5*b*. On the first day after the operation the headless implanted cockroaches showed a normal rhythm of activity. This disappeared during the second and third day, although in four experiments it continued for 4 days. The appearance of this normal rhythm after implantation was not altered by the light conditions; cockroaches were tested in continuous light and darkness, and in reversed light and darkness, and still showed the rhythm of activity previously shown by the donor of the sub-oesophageal ganglia.

Sub-oesophageal ganglia were implanted into entire cockroaches, one being placed in each. These cockroaches under conditions of alternating light and darkness continued to show a normal activity rhythm, but the level of the activity during the dark period was considerably lower than before the operation (Fig. 6*a*). There were present in the animals two sub-oesophageal ganglia after the operation, therefore as a control experiment sub-oesophageal ganglia from normal animals were implanted two at a time into headless cockroaches. No depression of activity was noted during the active period in these animals (Fig. 6*b*).

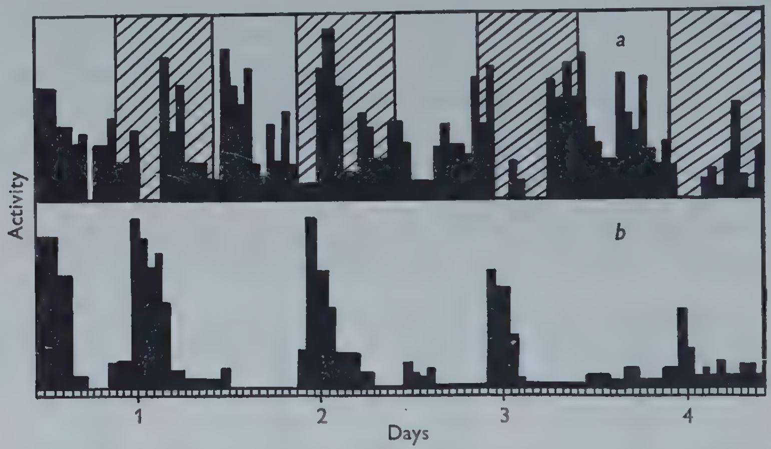

Fig. 5. (*a*) The activity of a headless *P. americana* in alternating light and darkness. (*b*) The activity in continuous light of a headless cockroach into which had been implanted the sub-oesophageal ganglia from a normally rhythmic cockroach.

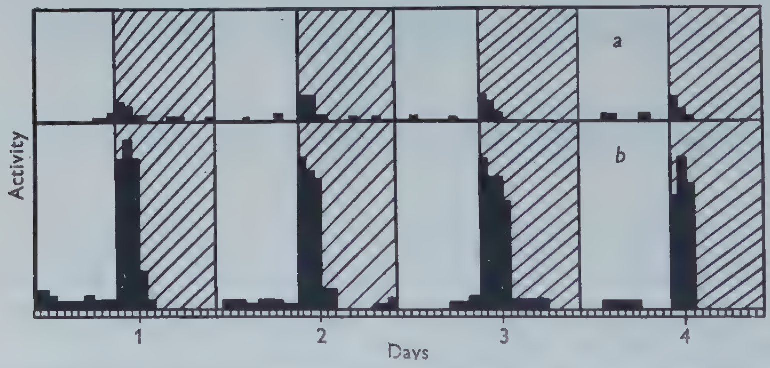

Fig. 6. (*a*) The activity of an entire *P. americana* into which had been implanted two sub-oesophageal ganglia from normally rhythmic cockroaches. (*b*) The activity of a headless cockroach into which had been implanted two sub-oesophageal ganglia.

Sub-oesophageal ganglia from animals with a normal rhythm were implanted, one per animal, into entire cockroaches which had been in constant light; that is, into cockroaches with the sub-oesophageal ganglia present, but which were not showing a rhythm of activity. These animals took up a rhythm of activity with a normal level of activity being reached in the active period.

The corpora allata were removed from animals with normal activity rhythms. This did not appear to affect the rhythm. When the corpora cardiaca were removed from normal cockroaches there was found to be no change in the rhythm while the cockroaches were in alternating light and darkness, but when placed in constant light there was no carry-over of the rhythm (Fig. 7). The total activity in the absence of the corpora cardiaca is considerably higher, however, than in a normal cockroach, and it is possible that this is masking any diurnal rhythm.

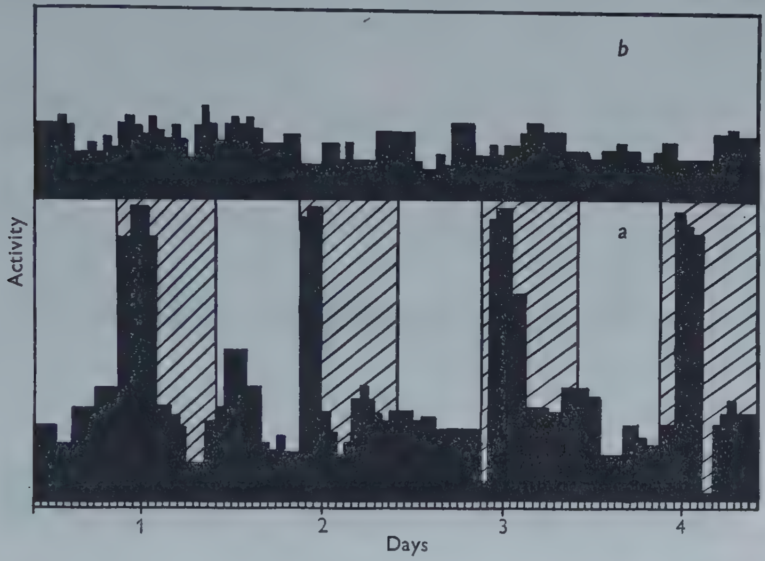

Fig. 7. The activity of *P. americana* after the removal of the corpora cardiaca (*a*) in alternating light and darkness, (*b*) in continuous light.

Other known endocrine glands, corpora allata, corpora cardiaca and brains were implanted into headless cockroaches, but in no case was implantation followed by any rhythmical activity as it was after implantation of sub-oesophageal ganglia.

Using the Gomori chrome-haematoxylin method neurosecretory cells were examined in the sub-oesophageal ganglia taken from cockroaches which had been subjected to continuous light or darkness or alternating light and darkness. No differences in their content could be detected.

(*c*) *The ocelli*

It would appear possible that the sub-oesophageal secretion might be governed by some sensory organ which is sensitive to light or darkness.

Cloudsley-Thompson has shown that blacking out the eyes and ocelli will cause a loss of rhythm. The compound eyes alone were covered in a group of ten animals which had previously been in constant light and were arrhythmic. They were then placed in alternating light and darkness and their activity measured. They all showed a normal rhythm after 2 days, and there was a carry-over of the rhythm when they were transferred back to continuous light. The optic nerves were cut in another series of animals from continuous light, and six out of ten animals showed a rhythm under alternating light and darkness, and this continued when the animals were removed to continuous light.

The ocelli alone were painted over in another series of animals which had been in alternating light and darkness. The cockroaches then showed a gradual loss of the original activity rhythm and an active phase appeared in the light period. The new rhythm was not one which could be carried over under new conditions. The peak of activity during the light phase was eliminated when the compound eyes were also blackened (Fig. 8).

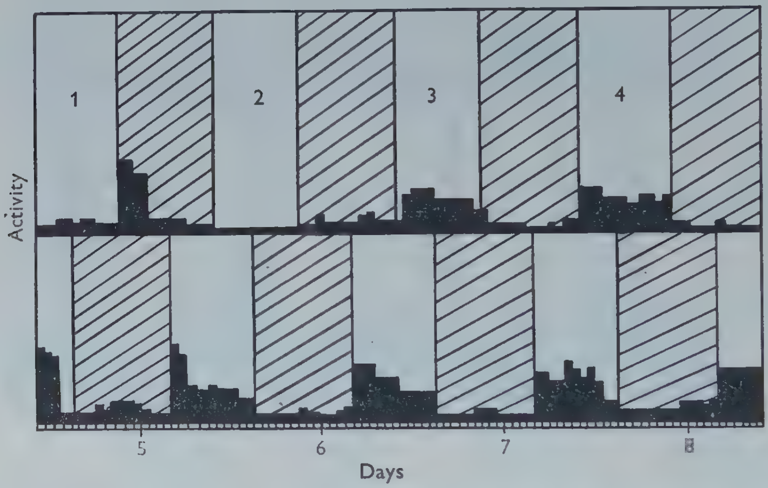

Fig. 8. The activity of *P. americana* in alternating light and darkness after the ocelli had been blackened.

SUBSIDIARY RHYTHMS

In the course of these experiments there has been observed another rhythm of activity subsidiary to the main diurnal rhythm. All the activity records of intact animals, and those of some of the operated or beheaded animals, show peaks of activity at regular intervals varying between 30 and 60 min. These are particularly obvious in the less active periods, but are not so evident when the animals are at the peak of their activity. This rhythm closely approximates to a similar periodicity in the oxygen consumption of resting cockroaches (Roeder, 1953).

DISCUSSION

The type of rhythm which has been studied in this work is one which can persist for a time without any reinforcement by the extrinsic conditions originally necessary for its establishment. That is, there appear to be concerned in the rhythm not only outside governing factors, but also an 'internal clock' with an ability to keep its own time.

It would seem fairly clear from the experiments that at least part of the mechanism of the internal clock is an endocrine secretion released by the sub-oesophageal ganglia, and that this is governed by the extrinsic factors of light and darkness through the medium of the ocelli. The sub-oesophageal ganglia themselves appear to be the 'retainers' of the rhythm in that they can be completely isolated (implantation experiments) and yet continue to secrete at definite times. This may indicate a rhythm of exhaustion of secretion, but so far experiments have revealed no differences in extracts taken from the sub-oesophageal ganglia at various times.

The mechanism through which the secretion acts is not known, nor is it by any means sure that activity occurs at the time of secretion. Should the secretion be inhibitory then the animal may be active only in its absence, and there is some evidence for it being inhibitory, at least in high concentration, under certain conditions. This is evident when the sub-oesophageal ganglia from normally active cockroaches are implanted into similarly active intact animals, the level of activity being considerably lowered in the subsequent active phases. On the other hand, there is no evidence of inhibition when two sub-oesophageal ganglia are implanted into a headless animal. Perhaps this indicates that other factors are involved in the intact animal in addition to a direct secretion from the sub-oesophageal ganglia, a view also supported by the results of the experiments in which the corpora cardiaca were removed.

If the secretion promotes the active phase, then, since activity continues for only a limited time in the dark period, either the secretion ceases to be effective or is exhausted, or a fatigue product is produced as the result of activity. This last possibility would also account for the slight increase in activity after about 6 hr. in light when the fatigue product would be decreasing.

Light acting through the compound eyes is probably stimulatory to a certain degree as was shown in experiments in which the ocelli alone were darkened. Cloudsley-Thompson (1953, p. 707) has shown that after a period of continuous darkness light causes a great increase in activity.

The reception by the ocelli of the extrinsic factor concerned in the rhythm may be taken as added evidence for the secretion taking place during the dark period (implying also that it causes activity). For Hoyle (1955) has shown that there is a continuous discharge of nerve impulses in the circum-oesophageal commissures when the ocelli are in darkness, and it is suggested that it is in this way that the sub-oesophageal ganglia receives its external control.

SUMMARY

1. By using a direct method of recording activity the diurnal rhythm of activity of *Periplaneta americana* L. has been investigated in detail under various conditions of light and darkness.

2. It has been found that there is no direct connexion between a hunger cycle and the activity rhythm.

3. By means of experiments in which cockroaches were joined together in parabiosis it has been shown that a rhythm can be relayed from one insect to another, and is postulated that a secretion carried in either the blood or tissues is responsible for the transmission.

4. Implantation of sub-oesophageal ganglia from cockroaches with a known rhythm into headless cockroaches has been found to result in the appearance of the known rhythm of activity in the latter.

5. Effects of other endocrine organs on the activity rhythm has been investigated.

6. The ocelli have been found to be directly connected with the establishment of the rhythm by the external factors of light and darkness.

7. A rhythm of activity subsidiary to the main diurnal rhythm is described.

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THE OSMOREGULATORY ABILITY OF THE LAMPERN (*LAMPETRA FLUVIATILIS* L.) IN SEA WATER DURING THE COURSE OF ITS SPAWNING MIGRATION

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(*Received* 19 *July* 1955)

INTRODUCTION

Lampetra fluviatilis (the lampern or river lamprey) undergoes a spawning migration in British rivers during the period between November and February, after which the animals spend from 4 to 6 months in fresh water prior to spawning. Although it is generally held that the earlier stages of the life history of the adult lampern are spent in the brackish water associated with the lower reaches of rivers, there is evidence that the animal can withstand much higher concentrations of sea water at this time, since Meek (1916) records the case of a metamorphosing lampern taken from the North Sea. The fact that lamperns are thus able to exist successfully in both fresh and salt water raises the question of the way in which osmoregulation is achieved in such diverse media.

During the spawning migration, the plasma osmotic pressure of the lampern remains relatively constant, measurements of freezing-point depression varying between −0·46 and −0·500° C. (Galloway, 1933; Dekhuyzen, 1904). Wikgren (1953) has demonstrated that the fresh-water osmoregulating mechanism is essentially similar to that already known for fresh-water teleosts (Krogh, 1939), and recently Robertson (1954) has shown that the ionic constituents of lampern plasma are present in similar proportions to those of a fresh-water teleost.

The only available record suggests that marine osmoregulation is accomplished by a hypotonic mechanism. Burian (1910) obtained a blood freezing-point depression of −0·58° C. from a single specimen of *Petromyzon marinus* (the sea lamprey) caught in the Mediterranean ($\Delta = 2{\cdot}3^\circ$ C.), and this value approximates to that quoted by Fontaine (1930*a*) for the same species migrating in fresh water ($\Delta = -0{\cdot}535^\circ$ C.). However, attempts to return migrating lampreys to sea water have failed to show the existence of a marine osmoregulating system either in *P. marinus* (Fontaine, 1930*a*, *b*) or in *L. fluviatilis* (Galloway, 1933). Both investigators found that lampreys showed raised blood osmotic pressures following immersion in sea-water solutions hypertonic to the blood of fresh-water animals, whilst sea-water concentrations of 50% and above were found to be lethal within 24 hr.

These observations imply that lampreys lose their powers of sea-water osmoregulation as they develop the fresh-water mechanism, the physiological basis for which is certain to be quite different. The present study is concerned with testing this hypothesis by comparing the marine osmoregulatory powers of lamperns caught as early as possible in their spawning migration (fresh-run animals) with others which had been kept in fresh water for much longer periods of time and were, in consequence, approaching maturity.

MATERIALS AND METHODS

Lamperns were obtained from the River Trent at intervals during the winter in sufficiently large numbers to make possible the comparative study of different populations.

Blood samples were collected from pithed animals by allowing blood to drain from the heart into heparinized hard-glass centrifuge tubes, chilled in ice. Particular care was taken to exclude both pericardial fluid and water from the samples.

The freezing-point depression (Δt) of sea water, plasma and urine samples was measured by Johlin's method (1931, 1933). A micro-Beckmann thermometer was employed to suit the determination of the small amounts of plasma available (0·7–1·2 ml.). Determinations on the same sample agreed within 0·005° C.

Plasma chloride was determined by the method described by Schales & Schales (1941). Using 0·2 ml. of plasma, duplicate samples gave variations of less than 2%. Urinary chloride was measured by the Volhard-Arnold technique, and sea-water chloride by the Mohr method using potassium chromate as indicator.

The conversion factor recommended by Krogh (1939) (293 mM $\equiv$ 1° C.) has been used to convert chloride concentrations into freezing-point depression (ΔCl). In making this conversion it has been assumed that an equivalent amount of monovalent cation accompanies the chloride.

A standard technique was used in determining the live weights of animals. Lamperns were taken from water with the bare hands and the water allowed to drain from the gill pouches before transferring the animal into a tared flask containing water. During transfer, the animals were allowed to slide between both hands to remove the water from the body surface.

Urine output was measured in one of two ways. In the first method, the head and trunk of the lampern were enclosed in a horizontal glass tube which opened into an aerated tank of water. The posterior end of the tube was closed by a rubber membrane which fitted around the body of the lampern. Urine was then collected by inserting the hind end of the animal into a glass test-tube closed anteriorly by a rubber membrane.

The second method of urine collection involved cannulating the urinary papilla by means of a fine metal or glass cannula after the animals had been anaesthetized in a saturated solution of chlorbutol. A rubber balloon was then fixed to the open end of the cannula and the whole firmly attached to the tail by means of thread. Lamperns recovered from the effects of the anaesthetic in 5 or 10 min. after being replaced in fresh water and were allowed to swim freely during the collection period.

All collections were made at a temperature of 16–18° C. over periods of time which varied from 6 to 24 hr.

THE RESPONSE TO A GRADUAL INCREASE OF ENVIRONMENTAL OSMOTIC PRESSURE

A gradual increase of sea-water concentration was obtained by allowing artificial sea water (Pantin, 1946) to drip at a constant rate into a tank of fresh water. Thorough mixing was achieved by aerating the tank, and the volume of water was maintained constant by withdrawing liquid from the surface by means of a vacuum system. Under these conditions, the rate of change of sea-water concentration is exponential and can be calculated from the expression

$$T_{(\alpha)}=2\cdot303\frac{V}{v}\log_{10}\frac{(1)}{1-\alpha},$$

where T is the time to reach a fraction α of 100% sea water, V is the volume of water in the tank (litres), v is the rate of inflow of sea water (litres/hr.).

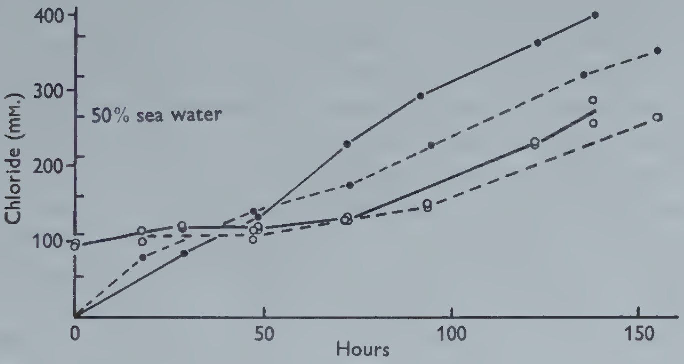


Fig. 1. The effect of gradually increasing sea-water concentration on the plasma chloride of *L. fluviatilis*. November experiment: O—O, plasma chloride; ●—●, sea-water chloride. anuary experiment: O - - - O, plasma chloride; ●- - - -●, sea-water chloride.

To avoid high rates of change of sea water during the early stages of the experiments, the rate of inflow was halved from that calculated to give a concentration of 80% sea water in 6 days, and was increased to the original value later. In this way approximately straight-line relationships between sea-water concentration and time were obtained (Fig. 1).

Measurements of the level of plasma chloride of individual animals and their ability to survive were used as measures of osmoregulating ability during experiments performed on fresh-run lamperns captured in November 1949 and on lamperns which had been kept in fresh water in the laboratory for 2 or 3 weeks (January 1951). Animals from the same groups, but kept in fresh water, yielded mean values of 96·8 ± 2·5 (from ten observations), 100·5 ± 1·6 (from seven observations) respectively for their plasma chlorides.

Though both experiments demonstrate that lamperns can only maintain their plasma chloride constant in mildly hypertonic environments (Fig. 1), there is evidence that fresh-run animals are able to withstand the effects of increasing sea-water concentrations better than more mature individuals, because the rapid rise in plasma chloride began at a higher sea-water concentration (230 mM Cl) than was the case in the more mature lamperns (150 mM Cl). In the later stages of both experiments, when the blood chloride was rising at about the same rate as that of the environment, the ventilation rate of the animals began to increase rapidly and they lay on their sides at the bottom of the tank, whilst the skin, fins and eyes showed a diffuse red tinge. The plasma obtained from these animals was heavily haemolysed and the red blood corpuscles were found to be markedly crenated. Those animals which were left at the end of the experiments soon died.

THE URINE OUTPUT AFTER ADAPTATION TO SEA WATER OF DIFFERENT SALINITIES

It was expected that the osmoregulatory ability shown by lamperns in the early stages of sea-water adaptation might be brought about, at least in part, by a reduction of urine output from the high fresh-water level. To investigate this possibility, measurements of urinary output were made on animals adapted to various concentrations of sea water for 2 or 3 days, and these are summarized in Fig. 2. Experiments were conducted mainly on fresh-run animals or animals which had been kept in fresh water for a few weeks after capture.

The volume of urine collected in fresh water shows marked differences depending upon the particular method employed. The high values of urine output obtained when the membrane method of collection was used (341·9 ± 41·6 ml./kg./day) are probably the result of damage to the delicate skin of the lampern by the membrane. Not only will this effect water uptake, but the irritation caused by the membrane will also bring about diuresis. Even after very careful handling, most lamperns show a loss of weight (Fig. 3*a*, 30 hr.; Fig. 4, fresh-water animals) which, in fresh water, can only be due to an increase of urinary output. Cannulation gives much lower and less variable results (155·8 ± 9·9 ml./kg./day) than the membrane method, indicating that these values are more likely to represent the normal urine output of the fresh-water animal.

The values obtained by other workers for the urine output of fresh-water lamperns are much higher than those obtained by the cannulation method. Wikgren (1953) obtained values of 359 ml./kg./day at 16–18° C., whilst Hardisty (1954) measured a mean increase of weight of 1·1 % per hr. after ligaturing the urinary papillae, a value from which a urine output of 264 ml./kg./day can be calculated. Though these figures resemble those obtained by the membrane method in the present studies and therefore suggest that diuretic responses may have influenced the results, there is a possibility that the higher urinary output may be attributable to an increase in the permeability of the external surfaces which, from evidence given later, appears to take place as the animal matures. Unfortunately, neither Wikgren nor Hardisty specify the particular stage their animals had reached.

The results of urine collections from animals kept in various concentrations of sea water show that urinary output is reduced in response to increased osmotic pressure (Fig. 2). In a similar series of experiments, Wikgren (1953) found that the volume of urine was reduced to 30–80 ml./kg./day when animals were kept in 100 mM NaCl (osmotically equivalent to 17·4% sea water), whilst in 200 mM NaCl (osmotically equivalent to 35% sea water) a urine output of 2·2–13·2 ml./kg./day was obtained. The higher values obtained by Wikgren may be due to the fact that collections were made without previously adapting the animals to the environment. Evidence is presented later that after abrupt immersion in sea water, the urinary output decreases gradually for a period of some hours, so that unless the animal has been adapted, a higher urinary output will be recorded.

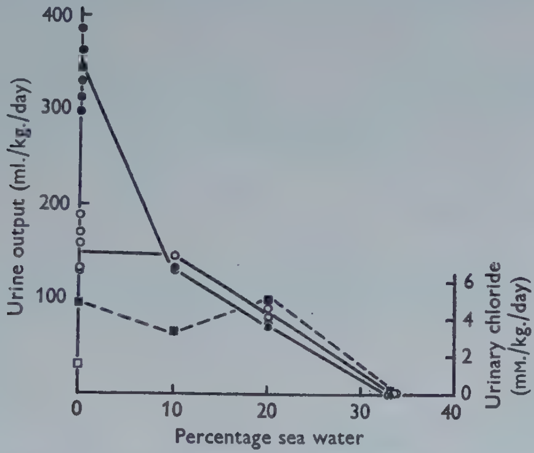


Fig. 2. The urine output and urinary chloride of *L. fluviatilis* kept in various concentrations of sea water. Urine output: ○, by cannulation method; ●, by membrane method. Urinary chloride: □, by cannulation method; ■, by membrane method.

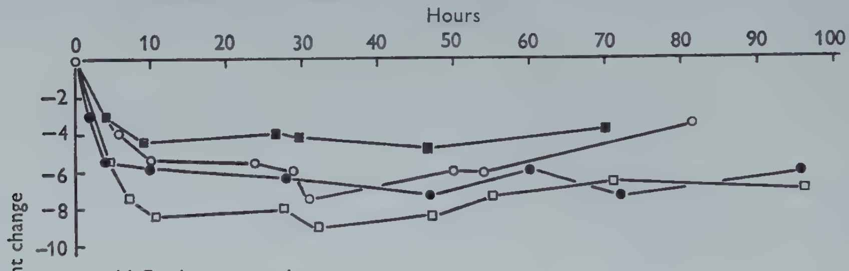
A comparison of measurements of urinary chloride from urine collected from fresh-water animals shows that, although the membrane method gives abnormally high results for chloride, there is little evidence that urinary chloride increases markedly with increasing sea-water concentration.

WEIGHT CHANGES AFTER ABRUPT TRANSFER TO SEA WATER OF DIFFERENT SALINITIES

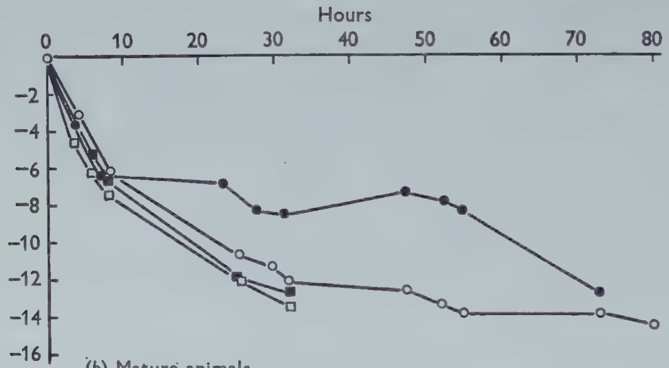
Serial determinations of weight were made on fresh-run and maturing lamperns in order to compare their ability to maintain a state of water balance in different environments. The migrating lampern does not feed, so that changes of weight are almost entirely due to changes of water content.

The weight changes were recorded from four fresh-run individuals immersed separately in fresh water, 33, 50 and 70% sea water. This experiment was repeated four times on different individuals within a short period of time, so that, in all, sixteen fresh-run animals were investigated. Later, the same procedure was applied to other animals which had almost reached sexual maturity in the laboratory (April 1952), although, since no experiments were performed in 70% sea water, only twelve maturing animals were involved. These lamperns were captured at the same time as those used in the earlier experiments and equal numbers of males and females were used. They were handled very carefully because of the danger of losing part of the content of the gonads.

The results of experiments performed on animals immersed in 33% sea water are given as typical examples in Fig. 3*a* and *b*.



(*a*) Fresh-run animals



(*b*) Mature animals

Fig. 3. The change in weight, expressed as a percentage of initial weight, of individual *L. fluviatilis* after immersion in 33% sea water.

Fig. 4*a* and *b* summarize the whole of the data collected during these experiments, and they have been constructed by interpolating values of percentage weight loss after 5 hr. and at subsequent 10 hr. intervals for each individual. The mean values of the weight change for animals treated in the same way (e.g. fresh-run animals kept in 33% sea water) have then been calculated for these particular time intervals, so that each line on the graph represents the mean percentage weight change with time for four individuals. The significance of the difference of means for particular time intervals (10 and 20 hr.) has been analysed statistically by the analysis of variance technique, and this has been expressed as the least significant difference and is included in Fig. 4*a* and *b*.

A consideration of Figs. 3 and 4 reveals that the animals can be divided according to the types of response they give. Some, consisting of nearly all fresh-run animals which had been immersed in 33 % sea water (Figs. 3*a*, 4*a*), gradually begin to gain weight after a period of high weight loss, whilst others, comprising the remaining fresh-run animals and almost all maturing animals, continue to lose water.

All animals show some loss in weight, particularly at the beginning of the experiments, for a variety of reasons.

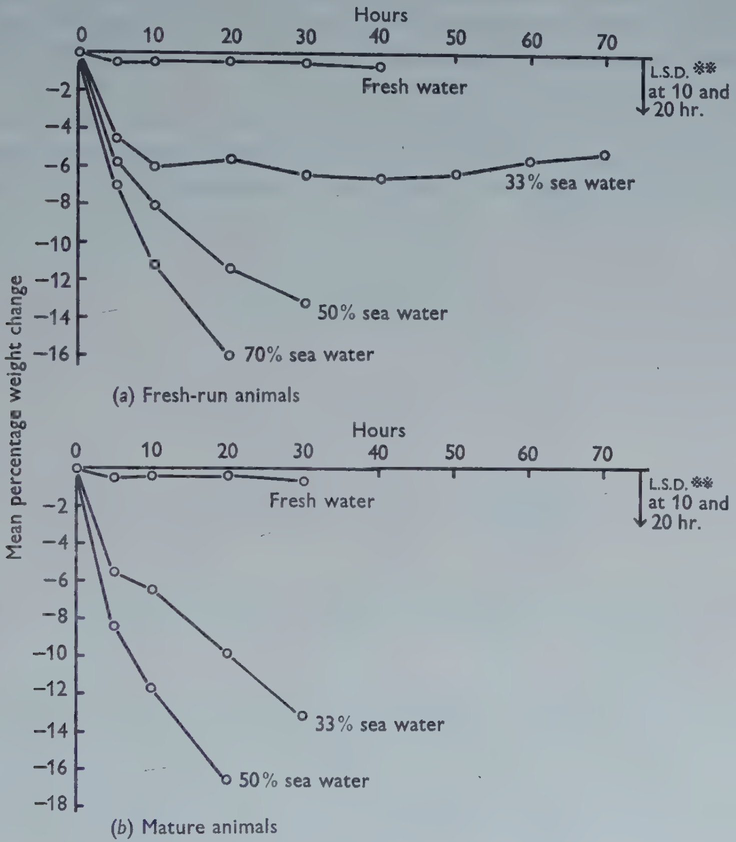


Fig. 4. The mean percentage weight change of *L. fluviatilis* after immersion in various strengths of sea water. (L.S.D.** is least significant difference.)

Diuresis caused by handling brings about some weight loss. This is shown clearly in both groups of fresh-water animals (Fig. 4*a*, *b*), where the loss of weight, in such a short time interval, can only be accounted for by an immediate increase in urine output. Individuals immersed in 33 % sea water (Fig. 3, 30 hr.) also show the same response.

Animals immersed in sea water also lose weight for other reasons. In all concentrations of sea water, animals show an initially high rate of weight loss which starts to decline between 5 and 10 hr. Although the initial rates of weight loss appear to be faster in more concentrated environments, the differences in the rates seem disproportionately small considering the wide range of sea-water concentrations in which the animals were immersed. The reason for this seems to be that urine output continues at the high fresh-water rate for some time and only gradually declines as the animal loses water. During an experiment designed to show this more directly, a lampern in fresh water gave a urine output of 170 ml./kg./day. For the first 12 hr. following immersion in 33 % sea water, the average urine output was 52·2 ml./kg./day, whilst immediately after this the urinary output amounted to 0·4 ml./kg./day. The expected weight loss after 12 hr. therefore becomes 2·61 % from this source, and this is within the limits of loss over this period for a fresh-run animal (Fig. 3).

Weight loss is also caused by the removal of water from the animal by the hypertonic external environment, and the rate at which this takes place will depend not only upon the osmotic gradient existing between the animal and the environment, but also upon the permeability of the tissues separating the two fluids. The effect of the osmotic gradient on the rate of water loss can be seen from Fig. 5. In this graph, the mean weight losses after 10 hr., for both fresh-run and nearly mature lamperns, have been plotted against the concentration of sea water responsible for the loss, and the figure will therefore include weight loss due to urine output as well as that caused by osmosis.

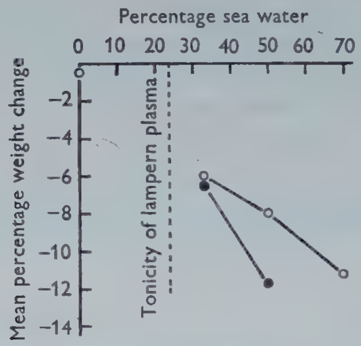


Fig. 5. The mean percentage weight loss of *L. fluviatilis* after 10 hr. immersion in various strengths of sea water. ○, fresh-run animals; ●, mature animals.

One must conclude from these experiments that only fresh-run animals immersed in 33 % sea water show any signs of maintaining a state of water balance, and that even in these animals this response is a variable one (Fig. 3*a*). The fact that in most of these cases the animal gains some weight in the face of so many factors tending to promote weight loss, indicates that some active process must exist which results in water being taken up by the animal.

Mature animals kept in the same environment fail to balance water in the same way (Fig. 3*b*), and, although they can exist for long periods of time in 33 % sea water, they eventually die. One lampern from this group actually gained weight at one stage of the experiment, but it eventually lost as much weight as the others.

CHANGES IN PLASMA CHLORIDE AND FREEZING-POINT DEPRESSION FOLLOWING IMMERSION IN 33% SEA WATER

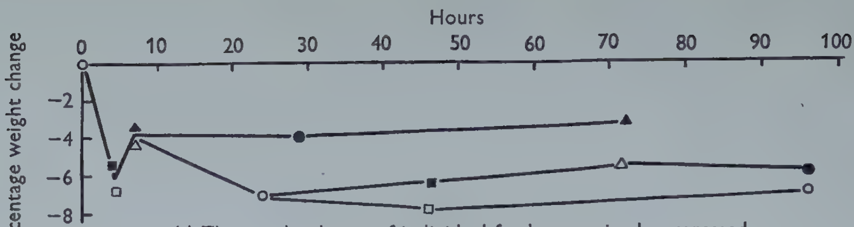
After it had been established that some fresh-run lamperns could only balance their water content in 33 % sea water, complementary experiments designed to assess ion balance were carried out on animals kept in this environment. The experiments were performed on groups of fresh-run and almost mature animals, which were removed in pairs from tanks of aerated 33 % sea water after a suitable length of time and measurements made of the levels of freezing-point depression and chloride in the plasma taken from each individual. In the case of fresh-run animals, the changes in weight brought about by immersion were also recorded.

The results of these experiments are illustrated graphically in Fig. 6*a*, *b* and *c*. In these figures the values for plasma chloride have been converted into freezing-point depression, and because of the need to relate plasma osmotic pressure (Δt), plasma chloride (ΔCl) and percentage weight change with time, a similar symbol has been used for each of the variables measured for any individual. The lines joining the points in Fig. 6*a* and *b* have been used as a means of grouping animals which appeared to respond in a similar manner and therefore may be without significance when used as an indication of the way in which a particular type of individual might behave with time.

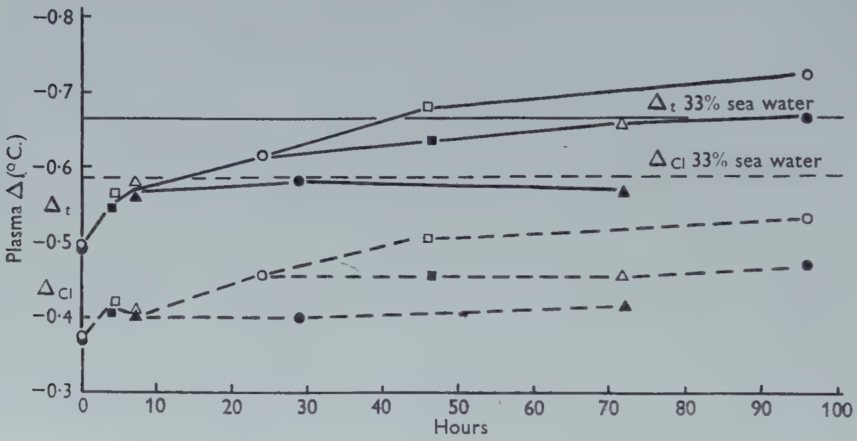
Two experiments were actually performed in April 1952, only one of which is shown (Fig. 6*c*). Almost the same responses were obtained in both cases, i.e. the plasma freezing-point depression and chloride rose at the same rate until at 40 hr. a steady state was attained.

Although it is clear that the osmoregulatory capacities of fresh-run animals are very variable (Fig. 6*a*, *b*), it is impossible to decide in the early stages of an experiment of this type whether low plasma osmotic pressure can be attributed to osmoregulatory ability, or to the short length of time during which the animals were immersed in sea water. For this reason, a second type of experiment was designed in which animals were immersed in 33 % sea water for a minimum period of 40 hr.; the length of time taken for animals to reach a steady state (Fig. 6*b*, *c*). Fig. 7 summarizes the results of several experiments of this pattern and also includes observations from fresh-run animals which were used in the previous experiments and which satisfy the same conditions. In this figure, the values of total osmotic pressure (Δt) and osmotic pressure attributable to chloride and its accompanying cation (ΔCl, shaded area) are plotted against the initial weight of the animal, which has been used as a measure of the size of the animal and hence its blood volume.

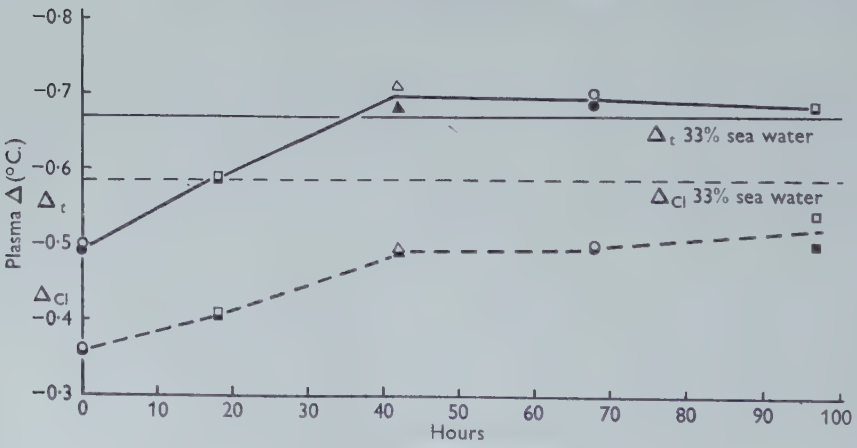
The figure emphasizes the fact that some fresh-run lamperns possess marked powers of osmoregulation in hypertonic environments, and also shows that this faculty is correlated with the maintenance of low blood chloride and slight water loss. In other animals caught at the same time, the ability to osmoregulate is present in different degrees, and some of these show large weight losses associated with plasma osmotic pressures which are hypertonic to their environment, so that they exhibit similar behaviour to mature animals kept under the same conditions.



(a) The weight change of individual fresh-run animals expressed as a percentage of the initial weight



(*b*) The plasma freezing-point depression (Δ_t) and plasma chloride (Δ_{Cl}) of the same fresh-run animals



(c) The plasma freezing-point depression (Δ_t) and plasma chloride (Δ_{Cl}) of mature animals

Fig. 6. The osmotic responses of fresh-run and mature *L. fluviatilis* after abrupt immersion in 33 % sea water. (For further explanation see text.)

DISCUSSION

These studies show that there are marked differences in the marine osmoregulatory capacities of fresh-run and mature lamperns, and that even fresh-run animals may vary considerably in their powers of osmoregulation.

Mature lamperns are incapable of maintaining ion or water balance in sea water hypertonic to their blood and, in consequence, are unable to osmoregulate. These animals show similar responses to those obtained by Keys (1933) in his work on the eel. He prevented eels from osmoregulating by blocking their swallowing mechanism, after which the animals lost water continuously and eventually died when they had lost 11–14 % of their total body weight. The lampern usually dies after losing 14–19 % of its total weight.

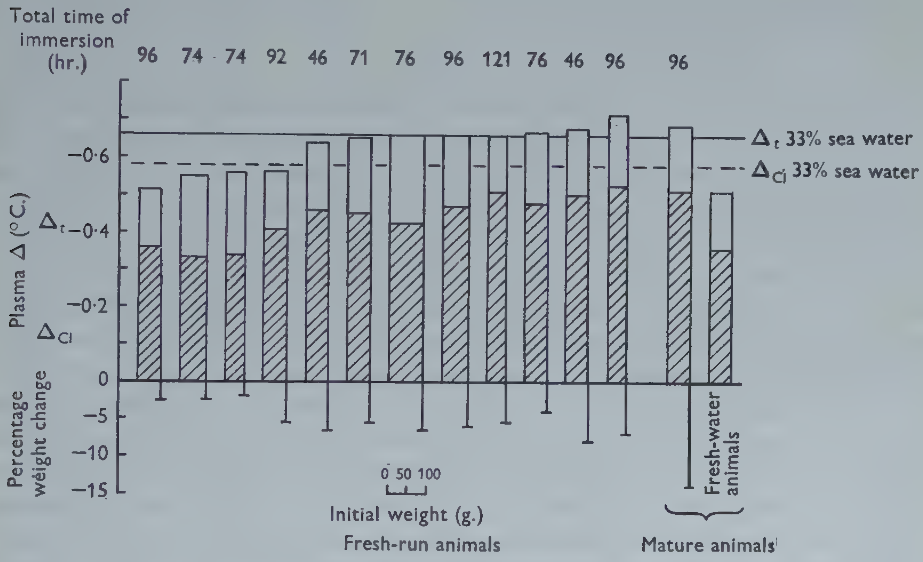


Fig. 7. The osmotic responses of individual *L. fluviatilis* after abrupt immersion in 33 % sea water for a minimum period of 40 hr. (For explanation see text.)

In 33 % sea water the curves relating plasma chloride and osmotic pressure with time (Fig. 6*c*) and weight loss with time (Fig. 4*b*) are similar in character, though inversely related; both indicate that a steady state is reached in about 40 hr. These observations suggest that water loss from the kidney and from the gills and integument is mainly responsible for raising the plasma osmotic pressure. As a steady state is approached, the plasma osmotic pressure eventually becomes higher than that of the surrounding sea water, whilst the plasma chloride remains somewhat lower. Osmotic water loss alone is insufficient to explain this situation, and though this condition could be brought about by the excretion of a hypotonic urine, the very small amount of urine produced by fresh-run animals in 33 % sea water (Fig. 2) would indicate that this is improbable.

Though fresh-run animals vary in the way in which they respond to sea water, some animals manage to osmoregulate efficiently in 33 % sea water; an environment which is hypertonic to the blood of the fresh-water lampern. The responses of these animals are characterized by a low blood osmotic pressure ($\Delta = -0{\cdot}55^\circ$ C.) which may be brought about by the active maintenance of the low blood chloride associated with it. There is thus no doubt that the lampern can carry out hypotonic regulation in 33 % sea water. This finding is in agreement with the measurement made on *Petromyzon marinus* by Burian.

Table 1. *A comparison of weight loss in the lampern and the eel*

Animal	Environment (% sea water)	Mean percentage weight loss after 10 hr.
Eel (Keys, 1932)	100	3–6
Lampern (fresh-run)	33	3·9–8·4
Lampern (fresh-run)	70	10·9–11·4

Those animals capable of osmoregulation in 33 % sea water respond in a similar manner to the eel when it is transferred from fresh water to full-strength sea water (Keys, 1933). Both animals show a passive phase of water loss which is followed by an uptake of water after a period of 30–40 hr. has elapsed. The most important difference between the fresh-run lampern and the eel lies in the strength of sea water in which they are able to survive, lamperns appearing unable to withstand sea-water concentration greater than 50 %, either after abrupt transfer or when gradually acclimatized to this concentration under the conditions of the present experiments. One of the reasons for the difference is that the lampern loses much more water than the eel before the active process responsible for water uptake intervenes (Table 1). These figures, though not directly comparable, indicate a marked dissimilarity between the water permeability of the external surfaces of the animals, since it seems unlikely that the disparity in the surface volume/weight relationship between the animals, or the differences of the rates of urine output, could account for such a large difference in the rates of initial water loss.

There is evidence that the external surfaces of the lampern become more permeable to water as the animal matures (Fig. 5), and it is interesting to speculate that this situation may be the end result of a process which starts at the very beginning of the migration, so that even in fresh-run animals capable of osmoregulating in 33 % sea water, the external surface permeability may have already increased sufficiently to make survival in more concentrated environments impossible. This argument receives some support from the fact that fresh-run animals show marked differences in the rates at which they lose water during the early stages of weight-loss experiments (Fig. 3*a*), indicating that these animals may have reached different stages in the general trend toward increased permeability.

One of the most striking features of nearly all the experiments conducted on fresh-run animals is that they show gradations of osmoregulatory ability which can only be explained by assuming that a gradual breakdown of the mechanisms which control sea-water osmoregulation takes place. The causes of this breakdown process cannot be analysed from the present experimental data, but it should be noted that at least three separate mechanisms may be involved. Besides the increase of water permeability already considered, there are signs that the mechanism responsible for the water uptake is also undergoing a marked change, because some fresh-run animals show little or no gain in weight (Figs. 3*a*, 7), whilst in the case of the mechanism controlling ion balance, there is a similar variability which is reflected in the levels of the plasma chloride of various individuals (Fig. 7). The details of the mechanism of marine osmoregulation and the causes of its breakdown will form the subject of a later communication.

SUMMARY

1. Although fresh-run lamperns are able to withstand the effects of increasing sea-water concentration better than maturing animals, they can only maintain their plasma chloride constant in environments more dilute than 50 % sea water. This is achieved, in part, by gradually reducing the urine output from the normal fresh-water level (155·8 ml./kg./day) to a negligible rate in solutions which are mildly hypertonic to the blood (33 % sea water).

2. Studies on the rate of change of weight loss, of plasma chloride and of plasma osmotic pressure following abrupt immersion in dilute sea water show that mature lamperns cannot osmoregulate and can only survive in 33 % sea water by tolerating a raised blood osmotic pressure caused by water loss.

3. Similar experiments on fresh-run animals suggest that the external surfaces of their bodies are less permeable to water than mature animals. Unlike mature animals, they also show considerable variation in the way in which they respond to 33 % sea water. Some are able to maintain their plasma osmotic pressure and chloride well below that of the environment. These animals also show little loss in weight, and this indicates that water is taken up actively, since this process has been shown to occur in some animals. Other fresh-run animals show raised plasma osmotic pressures in varying degrees and these are associated with larger losses of weight. These facts suggest that the hypotonic regulating mechanism gradually degenerates as the lampern enters fresh water.

This work forms part of a thesis submitted for the degree of Doctor of Philosophy to the University of Nottingham. I should like to thank Prof. E. J. W. Barrington for his valuable advice and criticism throughout the investigation. I am also indebted to Mr J. R. Mallard, at that time of the Physics Department at the University of Nottingham, for deriving the formula relating change of sea-water concentration with time.

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RED BLOOD-CELL ANTIGENS IN SOME LOWER VERTEBRATES

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INTRODUCTION

Following Landsteiner's discovery of the human ABO blood groups in 1900, many attempts were made to find blood groups in other animals. The investigations were confined mostly to mammals, but Fishbein (1913) made a brief mention of the fact that he had been unable to find blood groups, similar to the human ABO system, in frogs. This was confirmed by Skarzynska (1925), who found no evidence for blood groups in *Rana esculenta*, or in the fish, *Cyprinus carpio*. In addition, she was unable to induce the production of antibodies in either species by injecting red cells from another animal of the same species.

Antigens similar to the human A and B antigens are known to be of widespread occurrence among both plants and animals. Cushing & Sprague (1952) found an antigen, similar to the human B antigen in the fish *Genyonemus lineatus*, but not in *Cymatogaster aggregata*, which may however possess the Forssman antigen; species antibodies for both species were found in the human sera used for the tests. An antigen, identical with the human B antigen, is said to have been found in frogs by Japanese workers (Furuhata, 1947). Toads, but not frogs, have the Forssman antigen (Boyd, 1939).

The methods used in these early attempts to find blood groups in Amphibia and fish are rather dubious, since they are not fully described by the authors. It was decided to see if blood groups could be found among these animals using the human blood-grouping techniques. The reactions of the cells of several Amphibia with human sera were also investigated.

The notation for the human sera used in this paper is that proposed by Ford (1955).

METHODS

Taking the blood. (1) *Amphibia.* The most satisfactory method was to decapitate the animal and let the blood drip from the neck into a sterile bottle. The average amount so obtained from frogs was about 1 ml. If the red cells only were required, some anticoagulant (either acid citrate dextrose or heparin in the same concentrations as used for human cells) was put into the bottle before the blood was added. The blood was stored at 4° C. (2) *Fish.* The fish were much easier to bleed, due to the larger size of the heart. A heart puncture with a syringe usually yielded 1–3 ml.

of blood, depending on the size of the fish. The blood was treated in the same way as the amphibian blood.

Serum. When the serum was required, the whole blood was allowed to clot and was stored at 4° C. overnight to let the serum separate from the clot. The serum was then centrifuged to remove the free cells and stored at −15° C.

Cells. All the cells, whether obtained from a clot or from a sample of citrated or heparinized blood, were treated in the same way. They were washed three times in isotonic saline and then approximately 2 % suspensions of cells in saline were made.

Frog cells seemed to be preserved equally well in 0·9 % or 0·65 % saline, the former being the concentration used for human cells, and the latter being isotonic with their body fluids; either concentration was used for the suspensions. The fish cells were not so tolerant. 1 % saline was used; this seemed an average value since marine teleosts vary considerably in the salt content of their blood, e.g. between 0·10 M-NaCl and 0·26 M-NaCl (Green & Hoffman, 1953).

The cell suspensions were stored at 4° C. At this temperature they remained in a good condition for at least 4 days. Suspensions which had haemolysed were not used in the tests.

Tests. All the tests were done in precipitin tubes (2 by $\frac{1}{4}$ in.). Equal volumes (approximately 0·01 ml.) of cell suspension and serum were put into the tubes with a Pasteur pipette. After shaking the tubes to mix the cells and serum, they were left for 1–2 hr. The mixture was then placed on a slide and examined microscopically for agglutination.

In contrast to its effect on human antibodies, temperature seems to have little effect on the activity of animal antibodies. Therefore, as serum was scarce, the tests were incubated only at room temperature, which was 20 ± 4° C. The only exceptions were tests with anti-D(A), which were incubated at 37° C. for 2 hr.

Absorptions. The following method was used to remove an antibody from a sample of serum. Equal volumes of packed washed cells and serum were mixed and left for 1 hr. at an appropriate temperature, e.g. 4° C. for anti-G(A) and anti-G(B), etc. The mixture was centrifuged and the serum recovered. This was repeated with a fresh sample of the same cells, three or four times, until the antibody was completely removed.

Titrations. The amount of antibody agglutinating the cells may be determined by titration. Serial dilutions, in saline, from 1 in 2 to 1 in 1024, of the serum were made. The cells were then tested against the dilutions of serum in the way described for the other tests.

Temperature of frogs. It should be mentioned that before the frogs were bled, they were kept for at least 7 days at 20° C. The production of antibodies in a high titre in cold-blooded vertebrates is related to the external temperature. Frogs, injected with human red cells, and kept at 8–10° C. produced no antibody, while those kept at 22–27° C. produced a haemolytic antibody (Allen & McDaniel, 1937). The same is true of the production of antibodies to bacterial antigens in both fish and frogs (Bisset, 1947). After a week at 20° C., the titre of any iso-antibodies that may have been present should have increased.

RESULTS

The first experiments were to discover if the individuals within a species of Amphibia or fish can be divided into groups according to differences in their red-cell antigens and naturally occurring iso-antibodies. This involved testing the cells of each animal against the sera from as many other animals of the same species as possible. About sixty frogs (*Rana temporaria*), forty-three whiting (*Gadus merlangus*), thirteen pouting (*G. luscus*) and twelve plaice (*Pleuronectes platessa*) were used for these tests. No agglutination of red cells by the sera was observed, and so there was no evidence from these tests for the occurrence of blood groups in these species.

These results indicate that blood systems, comparable to the human ABO system, are not found in these species of Amphibia and fish. Since there were no reactions, there is no evidence for the presence of either antigens or iso-agglutinins. But the results do not exclude the existence of a blood-group system (similar to the human Rh system), in which antibodies are not present unless the animal has been injected with cells of another group. This possibility was investigated by injecting a series of frogs with red cells of other frogs.

Two batches of frogs, one from Cornwall and the other from near Oxford, were used. It was thought that there was a greater chance of finding antigenic differences if animals from different areas were used, since blood-group frequencies may vary considerably between different areas. A number of animals from each batch was bled and the cells made up into 2% suspensions in 0·65% saline. The surviving frogs were given injections of cell suspension into the dorsal lymph sac; each Cornish frog was injected with 1 ml. of suspension from an Oxford frog, and each Oxford frog with ½ ml. of suspension from a Cornish frog. A total of four injections on successive days were given. The animals were then left for 14 days after the last injection before being bled. Throughout the period of the injections, the frogs were kept at 21° C., for the reasons stated earlier.

Eleven samples of sera were collected from the injected frogs. These were tested against the cells of the same eleven animals and several more, making a total of twenty-four frogs. No agglutination was recorded, suggesting that no immune antibodies had been produced.

There remained the possibility that incomplete antibodies were present. Several tests have been devised for incomplete antibodies, but only the albumin method was used for the frog cells. The red cells were suspended in 20% bovine albumin and these suspensions tested against the sera in the same way as the saline suspensions. No reactions were recorded. This strongly suggested that no incomplete immune-antibodies were present in the serum of the frogs which had been injected, and also that there were no naturally occurring incomplete iso-antibodies, since these, too, would have agglutinated the cells under these conditions.

The cells of about eighty frogs (*Rana temporaria*) were tested with human anti-G(A), anti-G(B), anti-G(A+B) (i.e. anti-A, anti-B and anti-A+B) and AB serum. The cells from every animal were agglutinated by all the sera, but the reaction was noticeably stronger with the sera containing anti-G(B). This was verified by titrating the sera against the cells. With anti-G(A) and AB serum, a reaction was recorded to dilutions of about 1 in 8 to 1 in 16, but with anti-G(B) and anti-G (A+B), the reactions continued to dilutions of 1 in 256. Since AB serum, which contains neither anti-G(A) nor anti-G(B), agglutinates the cells, it seems probable that a species antibody, i.e. a human anti-frog antibody, is causing the agglutination. The same explanation will apply to the agglutination with anti-G(A); the titrations show that the strength of the antibody concerned in the reaction with anti-G(A) and AB serum is similar. The reactions with anti-G(B) and anti-G(A+B) are probably due in part to the species antibody, since this should be present in all human sera, but also to anti-G(B) reacting with an antigen similar to the human B antigen.

Anti-G(A) and anti-G(B) were absorbed with human A and B cells respectively, and then titrated against frog cells. The results with absorbed anti-G(A) were similar to those with unabsorbed anti-G(A), but with anti-G(B), the reaction now ceased at dilutions of 1 in 8 to 1 in 16, compared to 1 in 256 with unabsorbed serum. The agglutination occurring with absorbed anti-G(B) was probably due entirely to the species antibody; this shows that frog cells are agglutinated by the anti-G(B) in the unabsorbed serum.

These results were confirmed by absorbing anti-G(A), anti-G(B) and anti-G (A+B) with frog cells, pooled from several animals. The absorbed sera were then tested against human A_1, A_2 and B cells. The anti-G(A) and anti-G(A+B) agglutinated the A_1 and A_2 cells, but the anti-G(B) and anti-G(A+B) no longer agglutinated the B cells. Therefore, frog cells remove anti-G(B) from the human serum and so must possess a B antigen. Since the frog cells have absorbed the anti-G(B) completely, the frog B antigen must be very similar to the human B antigen.

A similar series of tests was done with the cells from twenty-five toads (*Bufo bufo*), twenty-five tree frogs (*Hyla arborea*) and seventeen newts (*Triturus cristatus*). The results with the toad and tree frog cells were very similar to those with frog cells. When the sera were titrated against the cells, reactions were still apparent with anti-G(B) and anti-G(A+B) at much greater dilution, than with anti-G(A) or AB serum. Similarly, the reactions with anti-G(B) absorbed with human cells, were very like those with anti-G(A). Thus it appears that both toads and tree frogs have a B antigen.

It was not possible to verify the results for tree frogs by absorbing human sera with their cells, because the animals have so little blood. However, anti-G(A), anti-G(B) and anti-G(A+B) were absorbed with toad cells. When these absorbed sera were tested against human cells, the cells were still agglutinated by the appropriate serum. The sera were titrated and tested against the cells to see if the toad cells had reduced the titre of the antibodies. The results indicated that the titre of both anti-G(A) and anti-G(B) had been reduced from 1 in 256 to 1 in 8, so that neither antibody had been completely eliminated. The most probable explanation for the result with anti-G(B) is that the toad B antigen is not identical with the human B antigen, and so removes only part of the antibody. The result with anti-G(A) is more difficult to explain. It is known that toads possess the Forssman antigen; if cells possessing this antigen are injected into a rabbit, lysins for sheep cells are produced. The Forssman antigen is similar to the A antigen and is known to reduce the titre of human anti-G(A). Thus, it is probably responsible for the reduction of the titre of anti-G(A) observed after absorbing with toad cells.

The results with the newt cells were rather different. The cells of each animal were agglutinated to the same extent by the serum of each of the four ABO groups. The reactions with anti-G(A) and anti-G(B), absorbed with human cells, were similar to those with unabsorbed anti-G(A) and anti-G(B). This suggests that newts have neither an A nor a B antigen, but that the cells are being agglutinated by a species antibody.

About forty suspensions of frog cells were tested against human anti-D(A), anti-Ag(M) and anti-Ag(N). With anti-D(A), an agglutination comparable to that with anti-G(A), was obtained; this was possibly due to the human anti-frog antibody. Anti-Ag(M) and anti-Ag(N) did not agglutinate the cells. These results are interesting because the anti-D(A) serum is human, but anti-Ag(M) and anti-Ag(N) sera are made in rabbits. Therefore, there is a human anti-frog antibody, but not a rabbit anti-frog antibody.

DISCUSSION

The results described agree with the results of previous workers that blood groups cannot be found in Amphibia and fish. Fishbein (1913) makes no mention of the species (which was probably *Rana pipiens*), number of frogs involved, or the methods used. Similarly, Skarzynska (1925) gives few details, except that the results were the same, whether the tests were done at room temperature or 6–7° C. She remarks that all the tests were performed in winter, which means that the titre of any antibodies present in the serum would be exceedingly low. By keeping the frogs at 20° C. for a week before bleeding them, the titre of any antibodies present in the serum should have increased sufficiently for a strong reaction to occur with the cells. However, no iso-agglutinins appear to be present in the frog serum. The number of frogs used is too small to exclude completely the possibility that blood groups occur in the Amphibia, especially since the frogs all came from Cornwall. But, if a dimorphism does occur among frogs, all those used must have belonged to one group, so the frequency of the other group must be low. However, if the less frequent group includes only 1 % of the population, this frequency is too high to be maintained in the population by recurrent mutation and so would indicate a true polymorphism.

Similarly, only small numbers of fish were used, and so again the possibility of blood groups is not excluded. But, as in the case of the frogs, the other groups would have a low frequency.

Unfortunately, it was only possible to inject a few frogs and so there remains a chance that red-cell antigen differences may still be found by this method. It was hoped that under the experimental conditions any incompatibility between the cells of the donor and those of the injected frogs would produce a reaction. The conditions of the earlier attempt to induce the production of antibodies in frogs and fish (Skarzynska, 1925) were not so favourable, as each frog had a single injection of

0·5 ml. of a 10% suspension of cells and the experiment was performed in winter, which suggests that the animals were not at a sufficiently high temperature to produce antibodies.

Skarzynska concludes that frogs and fish are unable to produce antibodies, but there is evidence that frogs (*R. pipiens* and *R. catesbeiana*) will produce a haemolytic antibody to human red cells, although only if kept at a high temperature (Allen & McDaniel, 1937). The production of antibodies to bacterial antigens is a similar process; it was found that both fish and frogs would produce antibodies to bacterial antigens if kept at 20° C. (Bisset, 1947). So, in Amphibia or fish, if there are antigenic differences between the red cells of the different animals of one species, antibodies should be produced after injection provided that the animals are kept under the appropriate conditions. The only conclusion to be drawn from the experiments here described is that all the frogs used had the same antigens on their red cells.

The tests with human sera should be mentioned. Frogs, toads and tree frogs appear to have a B antigen, whilst newts probably do not. This fact is interesting, since the frogs and toads belong to the Anura and the newts to the Urodela. These two groups diverged very early in the history of the Amphibia. Similarly, in determining the relationships of the Amphibia as shown by the serum antigens, Boyden & Noble (1933) found a closer relationship among the members of the Anura, than between the Anura and Urodela.

The B antigens possessed by the frog and toad are not identical. The human B antigen has three components, B_1, B_2 and B_3, and animal B antigens may possess one, two or all of these components, e.g. the rabbit has B_2 and B_3 (Friedenreich & With, 1933). Frog cells absorb all the anti-G(B) from the human serum, and so must contain the same components as the human B antigen; this agrees with the results of the Japanese workers (Furuhata, 1947) but no mention is made of the species of frog used for their experiments. On the other hand, toad cells do not absorb the antibody completely and probably lack one or more of the components. Anti-G(B) was not absorbed with tree frog cells and the similarity of their antigen to the human antigen cannot be assessed.

The only example of blood groups in cold-blooded animals appears to be in two species of turtle, *Chrysemys picta belli* and *Chelydra serpentina* (Bond, 1940). Forty-seven *Chrysemys* were tested and these formed three groups; only five *Chelydra* were used. The tests were repeated with several other species of turtle and also with snakes (Bond, 1939), but there was no evidence for blood groups. It may appear strange that the groups are confined to only two species of reptile among those studied, but as yet there is no evidence that *all* the species within a single vertebrate class either have, or have not, blood groups, that all species have blood groups, or that no species have blood groups. For example, whilst most mammalian species possess blood groups, there is no evidence, so far, that they occur in the guinea-pig (Boyd & Walker, 1934).

The evidence at present suggests that blood groups may have started to occur in the reptiles and so their possession is confined mainly to the warm-blooded animals, i.e. birds and mammals. Although the significance of the blood group antigens is unknown, they are important in producing haemolytic disease of the newborn. The naturally occurring disease is confined to mammals, but it can be induced artificially in chicks (Briles, 1948). The reason why blood groups should be confined to these classes of animals is not at present clear.

SUMMARY

1. It was not possible to find evidence for blood groups in frogs (one species) or in fish (three species).

2. Frogs could not be induced to make antibodies after injection with red cells from another frog.

3. Frogs, toads and tree frogs have a B antigen; newts probably have not.

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